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XXIII. OXIDATION OF ISOPYROOXODELPHONINE, DIHYDROISOPYROOXODELPHONINE, AND THEIR DESMETHYLANHYDRO DERIVATIVES

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Studies have been in progress to explore the possibilities of the oxidative degradation of delphinine and aconitine and their derivatives, and we wish to record certain results which have been obtained principally with isopyrooxodelphonine and its dihydro derivative (1). On attempting the chromic acid oxidation of the precursors before saponification, *i.e.* isopyrooxodelphinine and octahydroisopyrooxodelphinine, the reagent acted in a different manner and far less readily than after saponification. With dihydroisopyrooxodelphonine, ($C_{24}H_{37}O_7N$), there resulted in good yield the *saturated keto acid*, $C_{24}H_{35}O_8N$, in which the four methoxyl groups remained unaffected. Diazomethane yielded the *methyl ester* which was not obtained crystalline but was readily purified by sublimation. From the ester a crystalline *oxime* was prepared. All of the oxygen atoms are thus accounted for by the four methoxyl groups and the carboxyl, carbonyl, and lactam groups. The same substance was obtained but much less satisfactorily with permanganate in acetone solution.

The experience with the chromic acid oxidation of the unsaturated isopyrooxodelphonine proved more involved. The major direct oxidation product is an *unsaturated keto acid*, $C_{24}H_{33}O_8N$ ($[\alpha]_D^{25} = +48°$). In this substance the oxygen atoms have also been accounted for by four methoxyl groups, the carboxyl and lactam groups, and an apparently less reactive carbonyl group. The acid yielded a crystalline *methyl ester*. Attempts to prepare an *oxime* from the latter encountered difficulties since the reaction occurred less readily than with the above dihydro derivative. Further study of the preparation of the oxime was abandoned in view of the observation that the unsaturated acid was converted by hydrogenation to the above saturated keto acid, $C_{24}H_{35}O_8N$, obtained directly from dihydroisopyrooxodelphonine. The presence of the carbonyl group in the unsaturated acid is therefore certain.

A complication developed in the gradual transformation of the unsaturated acid to a neutral isomer, found to be an apparently *saturated ketolactone*, $C_{24}H_{33}O_8N$ ($[\alpha]_D^{26} = +74°$). This isomerization was facilitated in acid solution or by sublimation of the acid. The lactone was readily

saponified with dilute alkali or ammonia to the original unsaturated acid. All attempts to hydrogenate the lactone were unsuccessful. However, in the presence of ammonia, which caused saponification, hydrogenation to the above dihydroketo acid readily occurred. Since experiments to prepare an oxime were unsuccessful, the reactivity of the carbonyl group assumed to be retained in the ketolactone appears to have become greatly hindered.
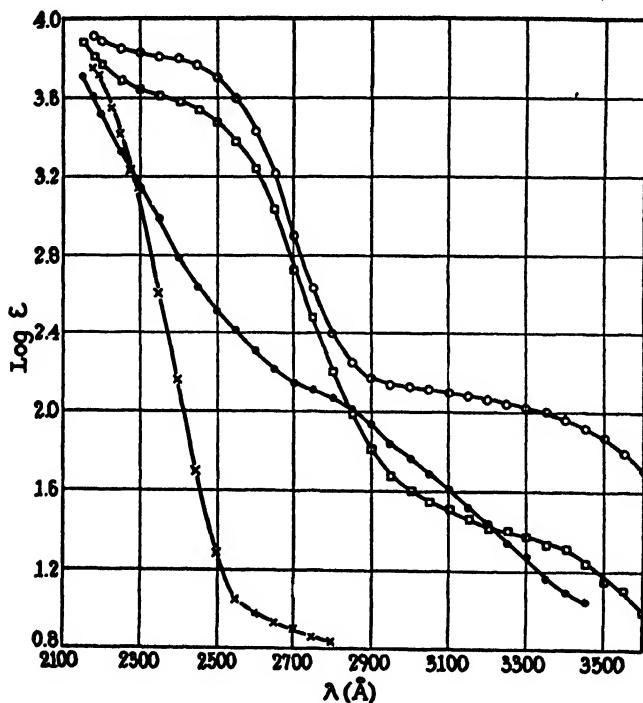
FIG. 1. ✗, isopyrooxodelphonine; □, the unsaturated keto acid, $C_{24}H_{33}O_5N$; ◯, the unsaturated keto acid methyl ester; ●, the saturated ketolactone, $C_{24}H_{33}O_5N$.

The ultraviolet absorption spectra curves obtained with these substances as shown in Fig. 1 have afforded some suggestions for their structural interpretation. As a possible reference the previously reported curve obtained from isopyrooxodelphonine (2) is repeated here. This shows end-absorption. The curve obtained with the methyl ester of the dihydroketo acid (Fig. 2) shows a band which can be attributed to the carbonyl group. The curves of the unsaturated keto acid and its methyl ester (Fig. 1), although the short wave maxima are low, might be suggestive of a $\Delta^{\alpha,\beta}$-unsaturated ketone. The curve (Fig. 1) of the saturated ketolactone $C_{24}H_{33}O_5N$ does not appear characteristic and the general absorption is such

as to mask carbonyl absorption alone. From the data obtained it is suggested that the unsaturated keto acid is of $\Delta^{\alpha, \beta}$-unsaturated ketonic character (Formula II) and, on isomerization to the saturated lactone, addition of the carboxyl group to the double bond occurs, as presented in the partial Formula III. On saponification of the latter, an unstable intermediate β-hydroxy ketonic structure may be first formed which loses water with the production of the original unsaturated keto acid.
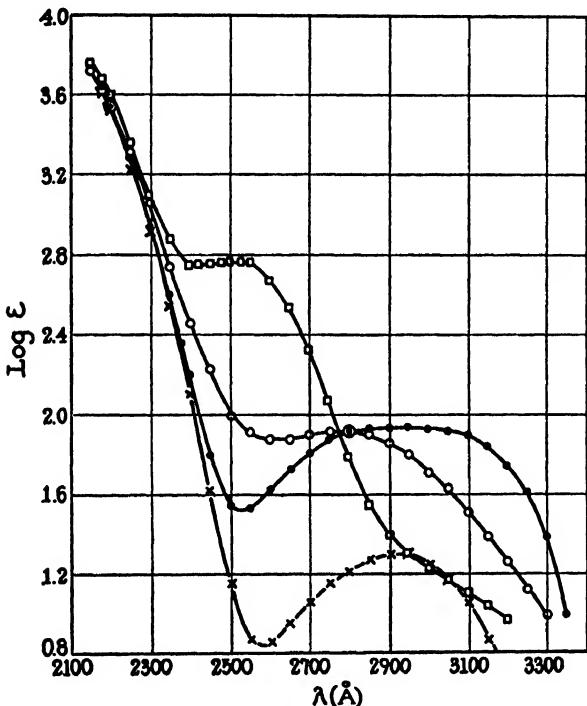
FIG. 2. O, the dihydroketo acid methyl ester; ●, the β-diketone; ×, the β-hydroxyketone; □, the monoketone (?), $C_{24}H_{27}O_6N$; all in ethanol.

The saturated keto acid, $C_{24}H_{35}O_8N$ (Formula IV), on sublimation behaves differently. It also yields a neutral substance but this involves loss of a mole of water. Analysis of the product indicated the formulation $C_{24}H_{33}O_7N$. The substance was found to be readily cleaved by alkali, not only with the regeneration of the original keto acid but with the formation of an apparently isomeric substance. A first conclusion which attributed the loss of water to lactonization on the enolized carbonyl group with formation of an unsaturated lactone was soon abandoned in favor of the interpretation that the condensation had caused the production of a β-diketone $C_{24}H_{33}O_7N$ (Formula V) of unusual lability towards alkali. On

hydrogenation of this substance in methanol, 1 mole of H_2 was absorbed with the production of a substance $C_{24}H_{35}O_7N$ (Formula VI). Contrary to its precursor, the latter did not consume alkali on attempted saponification. It has been provisionally interpreted as a *β-hydroxyketone* formed by hydrogenation of one CO group.[1] When the hydrogenation was performed in acetic acid, only a relatively small amount of the hydroxy ketone was obtained. The major product, as shown by the analytical data, resulted from a further loss of an oxygen atom. It appears possible that the hydrogenation proceeds in a different manner in acid solution with the formation of a *monoketone*, $C_{24}H_{37}O_6N$. The ultraviolet absorption spectra ob-

tained with these substances are shown in Fig. 2. The curve obtained with the so called monoketone, $C_{24}H_{37}O_6N$, is particularly unsatisfactory and this substance requires further investigation. The reason for the somewhat abnormal curves obtained with the β-diketonic derivative and its β-hydroxy ketonic hydrogenation product will also require explanation. The same curves were practically duplicated in later experiments.
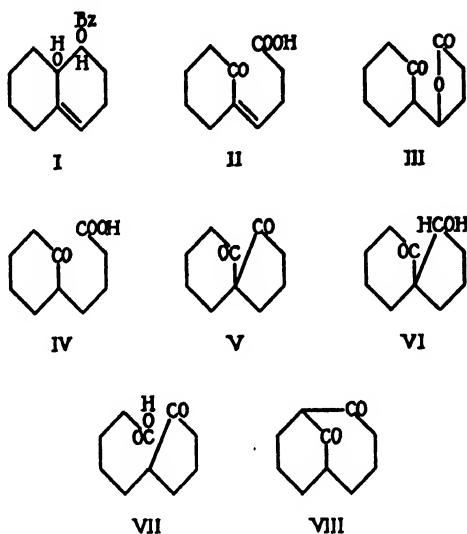
On the basis of these observations certain tentative conclusions may be summarized regarding the structural features of the alkaloid involved. Since oxidation of isopyrooxodelphinine and its hydrogenation product occurs readily only after saponification to yield keto acids, it is suggested

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[1] Since the hydrogenation of the unsaturated keto acid stops at the saturated keto acid stage (Formula IV), it would appear that its CO group is relatively resistant to hydrogenation. It seems therefore that the new CO group is the one reduced as shown in Formula VI.

that the original benzoyl group protects a secondary hydroxyl which adjoins a tertiary hydroxyl group, as indicated in partial Formula I. In the unsaturated keto acid $C_{24}H_{33}O_8N$ from isopyrooxodelphonine, the double bond of the latter is assumed to be in the position or has shifted to that shown in Formula II, α, β to the CO group. The carboxyl group would be properly oriented in regard to the $\Delta^{\alpha,\beta}$-carbonyl grouping to permit addition to the double bond with formation of the isomeric saturated keto-lactone, as shown in Formula III.[2] The latter when saponified could pass through the unstable β-hydroxyketo acid stage which loses water to form again the original unsaturated keto acid (Formula II). The latter in turn on hydrogenation yields the saturated keto acid (Formula IV) obtained directly by oxidation of dihydroisopyrooxodelphonine. It is assumed that loss of water from the latter on pyrolysis causes cyclization to form the β-diketone of spirane structure as presented in Formula V, which can in turn be hydrogenated to the β-hydroxy ketone (Formula VI).[1] The different ways in which such a diketone could be cleaved with alkali could explain the observations made. The point of rupture would determine either regeneration of the original keto acid or formation of an isomeric keto acid, as shown in Formula VII. The formation of a diketone as presented in Formula VIII has also been considered, since it might permit the other transformations observed, but it appears less likely. In all of these reactions the four methoxyl groups remain unaffected and there is no evidence that the lactam group participates in any of the transformations.

The apparent greater resistance to demethylation of the dihydroisopyrooxodelphinine series as discussed in the previous communication (2) has been substantiated by a study of the action of zinc chloride and HCl on the saturated keto acid, $C_{24}H_{33}O_8N$ above. Only two methyl groups and 1 mole of water were readily removed under the conditions used with the formation of the *dimethylanhydroketo acid* $C_{22}H_{29}O_7N$. The loss of water occurs presumably with formation of an oxidic ring and is restricted here to the hydroxyl groups exposed by the dimethylation, since they are the only ones available.

In the previous communication the oxidation of desmethylanhydroisopyrooxodelphinine was shown to yield a monoketo derivative. Following the above experience with isopyrooxodelphonine, it was of interest to study the oxidation of the saponification product of the former, desmethylanhydroisopyrooxodelphonine. The result appeared to parallel that obtained

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[2] An alternative but less favored interpretation would place the conjugated double bond in the first ring. The lactone which is formed would then be a less likely ϵ-lactone or a δ-lactone if the carboxyl group arises from cleavage of a cyclopentane ring. In the latter case appropriate changes would be required in the other formulas given.

with isopyrooxodelphonine, but with the additional oxidation of the secondary hydroxyl group exposed by the demethylation. After long continued extraction of the diluted reaction mixture with chloroform, two isomeric substances were obtained, one an acid and the other of neutral character. Analysis of these indicated the formulation $C_{20}H_{31}O_7N$. The acid has been interpreted as the *unsaturated desmethylanhydrodiketo acid* in analogy with the experience with the above tetramethoxy derivative. This is assumed to be the initial product which gradually and incompletely lactonizes on the double bond to the isomeric neutral *saturated desmethylanhydrodiketolactone*. The latter is readily saponified and, although gradually attacked by dilute ammonia or Na_2CO_3, it dissolves readily in fixed alkali. If these substances have been correctly interpreted, the original methoxyl groups after demethylation to hydroxyls are sufficiently removed from the assumed vicinal tertiary and secondary hydroxyl groups not to participate in the oxidative cleavage of the latter to CO and COOH.

In a preliminary study of the oxidation of the dihydro derivative, desmethylanhydrodihydroisopyrooxodelphonine, the expected *desmethylanhydrodihydrodiketo acid*, $C_{20}H_{33}O_7N$, was obtained. This product also required long continued extraction for removal from the diluted oxidation mixture.

It was of interest in this connection to attempt to limit the factors which determine the isomerism between the pyro-α-oxodelphinine and the isopyrooxodelphinine series. For this purpose the previously described octahydropyro-α-oxodelphinine ($[\alpha]_D$) $= -14°$) (1) was saponified to *dihydropyro-α-oxodelphonine*. The latter was not obtained crystalline but its oxidation with chromic acid paralleled that described above with dihydroisopyrooxodelphonine. A *dihydroketo acid*, $C_{24}H_{35}O_8N$, resulted and its melting point (131–136°) was lower than that of the above dihydroketo acid (149–154°). Although the substances from each source thus appeared to be different, the resemblance in the other properties studied was sufficiently close to render a conclusion in this respect difficult.

<div align="center">EXPERIMENTAL</div>

Isomeric Unsaturated Keto Acid and Saturated Ketolactone—1.1 gm. of isopyrooxodelphonine (1) dissolved in 35 cc. of acetic acid were gradually treated with 5 cc. of Kiliani chromic acid solution (53 gm. of CrO_3 and 80 gm. of H_2SO_4 in 400 cc. of H_2O). Within 10 minutes at room temperature the reagent was used up. The diluted mixture was treated with an excess of sodium acetate solution and then exhaustively extracted with chloroform. The washed extract was reextracted with an excess of dilute Na_2CO_3 followed by water. The aqueous extract after reacidification with

H_2SO_4 was reextracted exhaustively with chloroform. On concentration and drying *in vacuo* 0.7 gm. of resin resulted.

In preliminary experiments it was found that such material on standing, especially in solution, very gradually yielded crystalline neutral material. After several months the main resin had partly crystallized and, when treated with a small volume of methanol, became copious. The crystals were collected with methanol followed by ether and amounted to 0.127 gm. The substance was neutral and formed small stout tetrahedral or hexagonal prisms which melted at 234–235°. In dilute methanol solution with nitroprusside and either Na_2CO_3 or NaOH, a negative yellow color quickly developed.

$$[\alpha]_D^{26} = +74° (c = 0.45 \text{ in } 95\% \text{ ethanol})$$

$C_{24}H_{33}O_5N$. Calculated. C 62.17, H 7.18, OCH_3 26.79
Found. (a) " 62.05, " 7.22,
(b) " 62.63, " 7.09 " 26.40
(c) " 62.30, " 7.15

Although the substance was not directly soluble in dilute Na_2CO_3 or ammonia, it gradually dissolved in dilute NaOH, due to saponification.

8.96 mg. of substance suspended in 1 cc. of ethanol on direct titration against phenolphthalein slowly but progressively consumed 0.203 cc. of 0.1 N NaOH; calculated for 1 equivalent, 0.193 cc. On addition of excess reagent and refluxing for $2\frac{1}{2}$ hours, no further consumption was noted.

2.115 mg. of substance were refluxed in 0.1 cc. of ethanol and 0.1 cc. of 0.1 N NaOH for $2\frac{1}{2}$ hours and titrated. Found, 0.0475 cc.; calculated for 1 equivalent, 0.0456 cc.

On acidification of the titration solution to Congo red with dilute HCl, the mixture gradually crystallized on standing.

Found, C 62.38, H 7.25

The mother liquor from the above 0.127 gm. of lactone was concentrated to small volume and diluted with H_2O. A resinous suspension formed which, when warmed and seeded with the lactone, gradually crystallized. However, after several days aggregates of much larger crystals appeared. 0.17 gm. of this material was removed mechanically from the suspension of smaller crystals and proved to be the acid now to be described.

The remaining suspension of smaller crystals was carefully treated with ammonia as long as apparent solution occurred, and sufficient to be in slight excess. The undissolved fraction which was collected with water amounted to 48 mg. and proved to be the neutral lactone.

The filtrate after acidification with acetic acid and seeding gradually yielded compact pointed micro prisms. 40 mg. were collected with water. The substance was readily soluble in dilute Na_2CO_3 and ammonia and

under the microscope did not show a sharp melting point, apparently owing to partial transformation into the neutral lactone. About 30 per cent melted at 196–199°, about 50 per cent at 230–233°, and the remainder at 241–245°.

$$[\alpha_D^{25}] = +48° \ (c = 0.415 \text{ in } 95\% \text{ ethanol})$$

For analysis it was dried at 110° and 0.2 mm.

$C_{24}H_{33}O_6N$.   Calculated, C 62.17, H 7.18; found, C 61.84, H 6.93
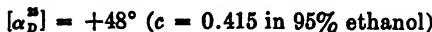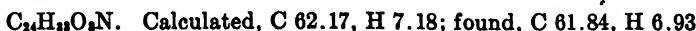
As in the case of the lactone, the acid showed no Legal reaction with nitroprusside.

30 mg. of the above acid were heated gradually in a sublimation apparatus at 0.03 mm. When the bath temperature reached 210°, most of the substance appeared to melt and at about 225° a deposit appeared on the condenser. During 15 minutes the bath was gradually raised to 275°. The sublimate was washed off with acetone and, when dried, weighed 26 mg. This crystallized readily in a small volume of methanol. This fraction weighed 9 mg. and was followed by an additional 5 mg. The substance was not soluble in dilute ammonia or Na_2CO_3.

$$[\alpha]_D^{25} = +74° \ (c = 0.42 \text{ in } 95\% \text{ ethanol})$$
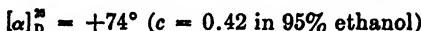
In other respects it proved identical with the above neutral lactone.

The above aqueous mother liquor from the acid fraction which had been acidified with acetic acid was now further acidified to Congo red with H_2SO_4. On long standing a final fraction of 35 mg. of neutral lactone was obtained which melted at 228–231°.

Methyl Ester—The previous acid (70 mg.) was treated with excess diazomethane in acetone. The concentrated solution crystallized as micro leaflets or rods. When crystallized from a small volume of cold ether, 52 mg. were obtained which gradually melted under the microscope at 197–202° with crystals remaining until 205°.

$C_{25}H_{35}O_6N$.   Calculated, C 62.86, H 7.39; found, C 62.68, H 7.21
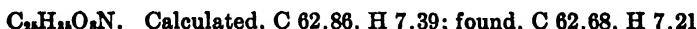
For the preparation of the *oxime* 20 mg. of ester were gently warmed to dissolve with a mixture of 12 mg. of hydroxylamine hydrochloride and 30 mg. of sodium acetate in 2 cc. of methanol. After several days, the concentrated mixture on careful dilution yielded 5 mg. of a crystalline fraction which contained unchanged starting material. The concentrated mother liquor on further manipulation yielded 7 mg. of a microcrystalline fraction which melted under the microscope at 173–178°. A small amount apparently of starting material persisted till 192–196°.

$C_{25}H_{36}O_6N_2$.   Calculated, N 5.69; found, N 5.04

Dihydroketo Acid, $C_{24}H_{33}O_5N$—0.3 gm. of dihydroisopyrooxodelphonine in 10 cc. of acetic acid was gradually treated at room temperature with Kiliani chromic acid solution. Following the addition of 0.9 cc. or about 2 equivalents of O, the reaction appeared more gradual. When a total of 1.2 cc. had been consumed, the mixture was diluted and thoroughly extracted with chloroform. The extract after washing with a small amount of water was concentrated finally *in vacuo* to remove excess acetic acid. The residue was redissolved in chloroform and the solution then reextracted with dilute Na_2CO_3. The latter was reacidified with H_2SO_4 and, in turn, thoroughly extracted with chloroform. The dried extract after concentration yielded 0.22 gm. of residue. The latter was dissolved in a small volume of methanol and after dilution gradually crystallized with about 1 mole of H_2O as stout nearly rhombic or rectangular prisms or platelets followed by some pointed blades. The acid melted gradually from 149–154° after preliminary sintering, especially above 140°. The melting point, however, varied considerably with the conditions of crystallization. From benzene it formed needles with or without solvent which sintered above 150° and gradually melted above 156°; but crystals persisted to 166°. It is sparingly soluble in H_2O and cold benzene, but readily soluble when warmed. It is soluble in alcohol and acetone.

$$[\alpha]_D^{28} = +7° \ (c = 0.98 \text{ in } 50\% \text{ ethanol})$$

For analysis it was dried at 110° and 0.2 mm.

$C_{24}H_{33}O_5N \cdot H_2O$. Calculated, H_2O 3.73; found, H_2O 3.73
$C_{24}H_{33}O_5N$. Calculated. C 61.90, H 7.58, OCH_3 26.67
Found. " 62.02, " 7.46, " 26.17

8.578 mg. of substance on titration with 0.1 N NaOH against phenolphthalein required 0.188 cc. Calculated for 1 equivalent, 0.185 cc. When boiled with excess reagent further consumption was negligible.

The oxidation with permanganate was much less satisfactory. 50 mg. of the dihydro derivative in 5 cc. of acetone were treated with 40 mg. of $KMnO_4$. Since there was no apparent reaction after 15 minutes, 0.05 cc. of acetic acid was added. Only a very gradual reaction occurred at room temperature and the reagent persisted for 2 days. The filtrate from MnO_2 was diluted and concentrated to remove acetone and the mixture was then made alkaline with dilute Na_2CO_3 and extracted with chloroform. The latter yielded 22 mg. of neutral residue which partly crystallized from acetone-ether when seeded with starting material.

The above Na_2CO_3 extract was acidified with H_2SO_4 and, after extraction with chloroform, yielded 31 mg. of a resin. This was dissolved in a drop of methanol followed by dilution. When seeded, crystallization

occurred and 12 mg. of substance were collected which proved identical with the above acid.

<p style="text-align:center">Found, C 61.93, H 7.54</p>

Methyl Ester—75 mg. of the previous acid in acetone solution were treated with excess diazomethane. Since attempts to crystallize the ester were unsuccessful, the resin obtained after removal of solvent was sublimed at 0.05 mm. pressure from a bath heated from 200–230°. A colorless resin was collected.

<p style="text-align:center">$C_{23}H_{37}O_8N$. Calculated, C 62.59, H 7.78; found, C 62.57, H 7.75</p>

1.863 mg. of substance were heated at 100° for $2\frac{1}{2}$ hours in 0.1 cc. of ethanol and 0.1 cc. of 0.1 N NaOH and titrated against phenolphthalein. Found, 0.0422 cc. Calculated for 1 equivalent, 0.0389 cc.

Oxime of Methyl Ester—20 mg. of the ester in methanol were heated for 1 hour with a mixture of hydroxylamine hydrochloride and sodium acetate. The concentrated mixture on dilution yielded 14 mg. of crystals. On progressive dilution of its concentrated methanol solution, the oxime crystallized as glistening 4-sided micro prisms or platelets which melted at 140–142°.

<p style="text-align:center">$C_{23}H_{33}O_8N_2$. Calculated. C 60.69, H 7.75, N 5.67
Found. " 60.87, " 7.74, " 5.95</p>

The relationship of the oxidation products from isopyrooxodelphonine and its dihydro derivative was shown in two ways.

0.275 gm. of isopyrooxodelphonine was oxidized with Kiliani solution as described above, and the chloroform extract was in turn extracted with dilute Na_2CO_3. The latter after acidification was reextracted with chloroform which yielded 0.15 gm. of a resinous acid product. The latter in methanol solution was hydrogenated with 50 mg. of platinum oxide. Although the H_2 absorption appeared complete in 2 hours, the operation was continued for 2 days. The apparent absorption in excess of the catalyst requirement was about 9 cc. This was in excess of the theory and possibly due to side reactions. The reaction mixture was diluted with chloroform, filtered, and the filtrate extracted with dilute Na_2CO_3. The chloroform phase was now found to contain an appreciable neutral fraction, since on concentration it yielded 89 mg. of residue. When treated with methanol, characteristic crystals of the neutral lactone separated. This fraction yielded 21 mg. and more separated from the mother liquor. It melted at 232.5–236.5°.

<p style="text-align:center">$C_{24}H_{33}O_6N$. Calculated, C 62.17, H 7.18; found, C 62.30, H 7.17</p>

Due to lactonization this fraction therefore escaped hydrogenation.

The above Na_2CO_3 extract after reacidification with H_2SO_4 was reextracted with chloroform. The latter yielded 62 mg. of a resin. The solution in a few drops of methanol, when carefully diluted, gave successive fractions of the dihydroketo acid as microtriangular leaflets which melted at 152.5°.

For analysis it was dried at 110° and 0.2 mm.

$C_{24}H_{35}O_5N$. Calculated, C 61.90, H 7.58; found, C 62.04, H 7.53

In another experiment 48 mg. of the unsaturated ketolactone, $C_{24}H_{33}O_5N$, were shaken in 95 per cent ethanol with 50 mg. of platinum oxide and H_2. After absorption of 12 cc. by the catalyst, no further absorption occurred even on heating to dissolve the sparingly soluble substance. The apparatus was then exhausted of H_2 and disconnected. A few drops of ammonia in sufficient excess were added; the apparatus was refilled with H_2 and the operation was continued. The crystals gradually dissolved, due to saponification, and H_2 absorption also became apparent. After 50 minutes the absorption (3.4 cc.) was somewhat in excess of 1 mole (about 2.5 cc.) and had practically stopped. The filtrate from the catalyst, after repeated concentration *in vacuo* to dryness, gave a residue of the free acid which, when dissolved in warm water and seeded, readily crystallized as micro prisms or leaflets which melted at 150–155° after preliminary sintering.

For analysis it was dried at 110° and 0.2 mm.

Found, C 61.71, H 7.59

β-Diketo Derivative—0.4 gm. of the dihydroketo acid was gradually heated in a sublimation apparatus with the pressure at about 0.01 to 0.05 mm. With the bath at 190° slight sublimation became apparent and the temperature was gradually raised during 10 minutes to 205° and then to 235°, when sublimation was practically completed. Following a final period the temperature reached 245°. The resinous deposit was dissolved in chloroform and the latter was extracted first with dilute Na_2CO_3 and then with water. The dried solution on concentration yielded 0.32 gm. of a neutral resin. The latter, when dissolved in acetone followed by addition of ether, crystallized as spear-headed plates or leaflets. 0.175 gm. was collected with ether. It melted at 141–142° after slight preliminary sintering.

$C_{24}H_{33}O_7N$. Calculated, C 64.39, H 7.44; found, C 64.34, H 7.40

In methanol or pyridine solution with sodium nitroprusside and dilute Na_2CO_3 it gave a red color, whereas the original keto acid showed a much more slowly developing reaction. With dilute NaOH there was less contrast and in both cases a deep red color developed.

The substance was readily cleaved with dilute alkali. 1.965 mg. of sub-

stance in 0.1 cc. of ethanol were titrated against phenolphthalein at room temperature with 0.1 N NaOH. It consumed the alkali gradually up to 0.0358 cc. Calculated for 1 mole, 0.0439 cc.

The ultraviolet absorption spectrum is recorded in Fig. 2.

As a check on its relation to the original keto acid, its alkali cleavage was studied as follows: 35 mg. of the substance were suspended in 1 cc. of H_2O and treated with excess ammonia. On stirring, and especially when warmed, it gradually dissolved. A small amount of ethanol was added with some additional ammonia and the mixture was repeatedly boiled down after H_2O addition to remove all ethanol and ammonia. When seeded with the keto acid, crystallization occurred. 5 mg. were collected which melted at 149–151° and were soluble in dilute ammonia. Manipulation of the mother liquor yielded about 1 mg. more. From benzene with ether it formed needles which sintered above 150° and gradually melted at 155–159° but not completely till 165°.

$$[\alpha]_D^{25} = +7.5° \ (c = 0.39 \text{ in } 50\% \text{ ethanol})$$
Found, C 61.58, H 7.66

The major portion remained in the mother liquor. Because of initial crystallization difficulties this was repeatedly extracted with chloroform and the concentrated solution was reextracted with dilute Na_2CO_3, followed by H_2O. Only a negligible fraction remained in the chloroform phase. The Na_2CO_3 extract was acidified with H_2SO_4 and repeatedly extracted with chloroform. The latter yielded 26 mg. of a resin. When dissolved in benzene and seeded with the keto acid, a mg. or so of the above substance crystallized. The mother liquor was concentrated to dryness. The residue dissolved in a little acetone followed by ether crystallized on standing as rosettes of flat needles or leaflets. 7 mg. were collected. It lost birefringence under the microscope about 75–80° and was definitely different from its precursor and probably isomeric. Its properties and the amount available made necessary a postponement of its further detailed study. For analysis it was dried at 100° and 0.2 mm.

$C_{24}H_{33}O_5N$. Calculated, C 61.90, H 7.58; found, C 61.30, H 7.45

β-Hydroxyketo Derivative—50 mg. of the above β-diketo derivative, when hydrogenated in methanol with 30 mg. of platinum oxide, absorbed slightly more than 1 mole of H_2 beyond the catalyst requirements. This occurred within an hour but the operation was continued for several hours. The concentrated filtrate yielded a resin which from a few drops of acetone on careful ether addition crystallized mostly as microtriangular prisms or platelets. The yield in this fraction was about 20 mg. A small additional amount was obtained from the mother liquor but much remained as a noncrystalline resin. This could not be crystallized when seeded with the

substance described below. After recrystallization from acetone-ether, the product melted at 212-213°.

$C_{24}H_{35}O_7N$. Calculated, C 64.10, H 7.85; found, C 64.07, H 7.78

When refluxed for several hours with a mixture of equal parts of methanol and 0.1 N NaOH, no consumption of alkali was noted.

The ultraviolet absorption spectrum is recorded in Fig. 2.

70 mg. of the diketone were hydrogenated with 50 mg. of platinum oxide in acetic acid. Although most H_2 absorption occurred during the 1st few hours, the operation was continued overnight when the apparent absorption beyond the catalyst was 10 to 11 cc. or a possible 3 moles. Chloroform was used during filtration from the catalyst and the filtrate was concentrated in vacuo to dryness. A solution of the residue in chloroform, when extracted with dilute Na_2CO_3, yielded negligible acidic material. The neutral fraction was obtained as a resin on removal of the chloroform and yielded from a concentrated acetone-ether solution a minor initial fraction. When collected with ether, 4 mg. were obtained. This together with a fraction from a second experiment was recrystallized from acetone-ether. It separated as small microtriangular or hexagonal prisms which agreed in properties with the above substance.

Found, C 64.13, H 8.06

The major product in the mother liquor was obtained in several fractions from ether in a yield of 40 to 50 mg. It formed micro platelets which melted at 168-170°. It gave practically no Legal test and absorbed no alkali on direct titration or after heating several hours with 0.1 N NaOH.

$C_{24}H_{37}O_6N$. Calculated. C 66.16, H 8.57, OCH_3 28.51
$C_{23}H_{35}O_6N$.   "   " 65.51, " 8.37, "   29.44
            Found. (a) " 65.56, " 8.46, "   28.50
                   (b) " 66.04, " 8.57
                   (c) " 66.01, " 8.58
                   (d) " 65.49, " 8.20

The ultraviolet absorption spectrum obtained is shown in Fig. 2.

Dimethylanhydroketo Acid—50 mg. of the dihydroketo acid, $C_{24}H_{35}O_6N$, were gradually dissolved in a solution of 2 gm. of zinc chloride in 0.7 cc. of 5 per cent HCl at 40°. After 45 minutes the clear diluted solution was extracted continuously with chloroform for several hours. The extracted resin crystallized readily from methanol as small hexagonal micro platelets or larger needles which melted at 272-277° after preliminary sintering.

For analysis it was dried at 110° and 0.2 mm.

$C_{23}H_{31}O_7N$. Calculated. C 62.97, H 6.97, $2(OCH_3)$ 14.80
            Found. (a) " 62.72, " 6.93,   "   · 15.60
                   (b) " 62.18, " 6.81,   "   15.10
                   (c) " 63.42, " 7.14,   "   15.41

2.012 mg. of dried substance on direct titration with 0.1 N NaOH against phenolphthalein required 0.0468 cc. Calculated for 1 equivalent, 0.048 cc.

Oxidation of Dihydropyro-α-oxodelphonine—Since dihydropyro-α-oxo-delphonine has not been crystallized, the amorphous material obtained by saponification of octahydropyro-α-oxodelphinine (1) was used for the oxidation. For this purpose a solution of 0.1 gm. of the latter in 2.5 cc. of methanol was treated with 1.2 cc. of 10 per cent NaOH. The clear solution after several hours at room temperature was treated with saturated NaCl solution and repeatedly extracted with chloroform. The extract was washed with NaCl solution, dried, and concentrated *in vacuo* to a resin. The latter in a mixture of 3 cc. of acetic acid and 0.3 cc. of H_2O was treated with 0.5 cc. of Kiliani solution. After 45 minutes at room temperature, the diluted mixture was extracted with chloroform in a continuous extractor. The extracted material after drying *in vacuo* weighed 80 mg. A solution in a few drops of methanol followed by H_2O addition gradually yielded a crust of crystals. 29 mg. were collected with H_2O. For recrystallization it was dissolved in H_2O with a slight excess of ammonia, cleared with norit, and the filtrate acidified with acetic acid. It crystallized as 4- or 5-sided boat-shaped or often triangular micro leaflets which melted at 131–136° after preliminary sintering. The mixture with the dihydroketo acid described above melted at an intermediate point, 143–147°.

$$[\alpha]_D^{\text{·}} = +2° \ (c = 0.79 \text{ in } 50\% \text{ ethanol})$$

$C_{24}H_{35}O_5N \cdot H_2O$. Calculated, H_2O 3.73; found, H_2O 3.32, 3.03

$C_{24}H_{35}O_5N$.   Calculated.   C 61.90, H 7.58
              Found.   (a)   " 62.06, " 7.84
                        (b)   " 62.00, " 7.61

Isomeric Unsaturated Desmethylanhydrodiketo Acid and Saturated Diketo Lactone—0.15 gm. of desmethylanhydroisopyrooxodelphonine was dissolved in a mixture of 0.75 cc. of H_2O and 2.25 cc. of acetic acid and gradually treated with 0.96 cc. of Kiliani solution. The reaction became more gradual after 5 minutes. After 25 minutes the diluted solution was treated with 0.7 cc. of saturated sodium acetate and the mixture was continuously extracted with chloroform for 18 hours. 31 mg. of a resin were extracted. After 24 hours more, a second fraction of 31 mg. followed which was succeeded in turn after 48 hours by 32 mg. A final 72 hours yielded 12 mg. The combined fractions in a small volume of water gradually crystallized, especially when crystals were once obtained. This was collected with a small volume of H_2O and weighed 51 mg. It was found to be a mixture of apparently neutral and acid material. For separation the suspension in a little water was carefully treated with ammonia

in slight excess and after thorough mixing the undissolved neutral lactone was collected with a little water. This material which amounted to 16 mg. was not directly soluble in dilute ammonia or Na$_2$CO$_3$, but rather quickly in dilute NaOH. It melted gradually over a range of 298–312° after some preliminary sintering about 255–257° and contained solvent. For analysis it was dried at 110° and 0.2 mm.

C$_{20}$H$_{21}$O$_7$N. Calculated, C 61.99, H 5.47; found, C 62.00, H 5.64

For recrystallization this material with a similar fraction from another experiment was dissolved in acetone-H$_2$O (5:1) and concentrated to remove most of the acetone. The solution slowly crystallized as needles. The substance after collection with water was still not directly soluble in ammonia or dilute Na$_2$CO$_3$. It partly softened apparently in solvent at 243° and finally melted at 313–316°.

Found, C 61.53, H 5.56

2.045 mg. of dried substance suspended in 0.1 cc. of ethanol and 0.1 cc. of H$_2$O were titrated directly with 0.1 N NaOH against phenolphthalein. The alkali consumption was gradual and after complete solution of substance amounted to 0.0524 cc. Calculated for 1 equivalent, 0.0528 cc. When heated for 2 hours with excess alkali, no further consumption occurred.

The ammoniacal filtrate from the neutral fraction was acidified with acetic acid and, when rubbed, the acid gradually crystallized as micro platelets which were mostly rhombic or derived therefrom. 21 mg. were collected with water. It was readily soluble in dilute ammonia and Na$_2$CO$_3$ and melted at 301–304°. It contained solvent. For analysis it was dried at 110° and 0.2 mm.

C$_{20}$H$_{21}$O$_7$N. Calculated. C 61.99, H 5.47
Found. (a) " 62.00, " 5.38
(b) " 62.10, " 5.49

A suspension of 1.97 mg. of dried substance in 0.2 cc. of H$_2$O and 0.2 cc. of ethanol was titrated directly with 0.1 N NaOH against phenolphthalein. Found, 0.049 cc.; calculated for 1 equivalent, 0.051 cc. When heated with excess alkali for 2 hours, no further consumption occurred.

Desmethylanhydrodihydrodiketo Acid, C$_{20}$H$_{23}$O$_7$N—The following account records a preliminary study in which the desmethylanhydrodihydroisopyrooxodelphonine used was the total resinous product obtained on hydrogenation of desmethylanhydroisopyrooxodelphonine (2) and before crystalline material had become available. It was possibly a mixture of isomers. 0.2 gm. dissolved in 1 cc. of H$_2$O and 3 cc. of acetic acid was gradually treated with Kiliani solution which was consumed within ½ hour. Con-

tinuous extraction of the diluted mixture with chloroform required an unusual time for completion. The approximate amounts extracted were 30 mg. after 22 hours, followed in succession by 25 mg. after 24 hours, 30 mg. after 24 hours, and 36 mg. after a final 72 hours. A diluted acetone solution of the second fraction on slow evaporation crystallized when nearly dry and the other fractions could then be seeded. The last three fractions were combined by solution in H_2O-acetone (1:9), filtered, and then concentrated repeatedly with dry acetone to remove most of the water. It slowly separated as a microcrystalline powder which was aided by addition of ether. After long standing this was collected with moist acetone in which it was appreciably soluble. It was readily soluble in H_2O and melted at 222-223°. For analysis the substance was dried at 110° and 0.2 mm.

$C_{20}H_{23}O_7N$.   Calculated, C 61.67, H 5.96; found, C 61.27, H 5.99

All the quantitative data have been obtained by Mr. D. Rigakos of this laboratory.

SUMMARY

Oxidation of dihydroisopyrooxodelphonine with chromic acid yields a keto acid, $C_{24}H_{35}O_8N$. The unsaturated isopyrooxodelphonine is oxidized to an unsaturated keto acid, $C_{24}H_{33}O_8N$, which can be hydrogenated to the previous acid. Contrary to the latter, the unsaturated keto acid isomerizes with acid or on distillation to a saturated ketolactone, $C_{24}H_{33}O_8N$, which cannot be hydrogenated except when again saponified. The production of the keto acid is interpreted as due to cleavage of vicinal tertiary and secondary hydroxyl groups. The latter occurs as the benzoyl ester in the parent alkaloid. The saturated ketolactone on distillation yields a β-diketo derivative, $C_{24}H_{33}O_7N$, the hydrogenation of which has been studied. The results of the oxidation of the demethylated anhydro derivatives are also described.

The structures involved in these reactions have been discussed and the conclusions drawn.

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THE VERATRINE ALKALOIDS

XXX. A FURTHER STUDY OF THE STRUCTURE OF VERATRAMINE AND JERVINE

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As has been reported previously (1), the most satisfactory interpretation of the major structural features of jervine, $C_{27}H_{39}O_3N$, is that it is a 3(β)-hydroxy-11-keto-$\Delta^{5, 8 (9)}$-steroidal secondary base in which the nitrogen atom is cyclized with carbon atoms of the isooctyl side chain. The 3rd oxygen atom appears to be of oxidic character and one point of its attachment must also be to a carbon atom of the side chain. This last conclusion was based on the isolation, from the dehydrogenation products, of a phenolic base, $C_8H_{11}ON$, which was suspected to be 3-methyl-6-ethyl-5-hydroxy-pyridine (2-ethyl-5-methyl-3-hydroxypyridine) (2) (Formula IV). The correctness of this interpretation has since been strengthened by more recent work.

Accompanying jervine in *Veratrum viride* is the secondary base veratramine, $C_{27}H_{39}O_2N$ (3, 4). The latter contains two easily acylatable hydroxyl groups, and a double bond which can be readily hydrogenated to yield a dihydroveratramine. Studies of absorption spectra of the alkaloid and its dihydro derivative have shown that one of its rings should be benzenoid and that one of the angular methyl groups has therefore shifted in the formation of the alkaloid. Both veratramine and jervine also occur conjugated as glycosides which have been isolated in the form of the glucosides veratrosine and pseudojervine (5). In the case of the latter, only the 3(β)-hydroxyl group, demonstrated to be present in jervine, can be involved in the conjugation and presumably in the β configuration. As previously reported, the attempt to show the possible presence of a 3-hydroxyl group in veratramine with aluminum *tert*-butoxide was unsuccessful. However, the presence of such a hydroxyl is to be inferred as the probable group involved in conjugation with glucose in veratrosine. Although a comparison of the rotations of the bases and their glucosides does not lend itself to the successful application of molecular rotation differences, as used by Barton (6) for simpler structural changes, nevertheless there is suggestive resemblance in the direct comparison of the rotations. Thus, on passing from jervine of $[\alpha]_D = -147°$ in ethanol to pseudojervine in ethanol-

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chloroform (1:3), $[\alpha]_D = -133°$. On passing from veratramine (
$-69°$ in methanol to veratrosine in ethanol-chloroform (1:1), $[\alpha]_D$

A preliminary study of the dehydrogenation of veratramine
lenium has shown that the second hydroxyl group of veratramine, as in the
case of jervine, is also on the basic side chain and the evidence is very strong
that it is on carbon atom 23. The dehydrogenation proceeded quite
smoothly, but the fraction of most volatile simple bases appeared to e
much less than has been the experience with other veratrine bases. The
yield of this fraction was too small from the amount of alkaloid used to
permit an attempt to isolate any methylethylpyridine, if formed. How-
ever, from the less volatile sublimate above the reaction melt an appreciable
amount of a phenolic basic fraction was obtained, from which in turn a
crystalline phenolic pyridine base was isolated. Analysis of this substance
showed it to possess the formulation C_8H_7ON. The colors given with fer-

ric chloride and diazotized sulfanilic acid were almost identical with those
obtained with the phenolic base $C_8H_{11}ON$ from jervine. The simplest
interpretation of this substance is that in its formation only the terminal
methyl (carbon 27) of the side chain is retained and that it is a hydroxy-β-
picoline. There was no evidence of the formation of the $C_8H_{11}ON$ base ob-
tained from jervine, which must be a 3-methyl-6-ethylhydroxypyridine.
From the nature of these substances as well as from the properties of the
parent alkaloids, the problem of the position of the hydroxyl is left to a de-
cision between a β- or γ-hydroxypyridine structure involving carbon atom
23 or 24.
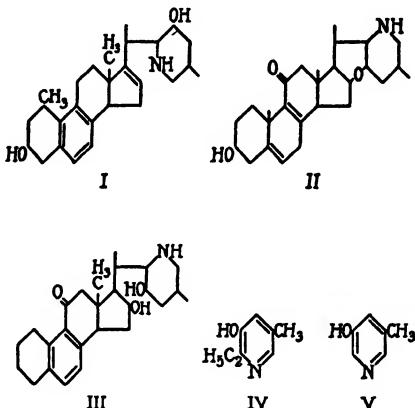
The results of a study of the ultraviolet absorption spectra of the sub-
stances from veratramine and jervine, which were taken in methanol and
in H_2O at pH about 1.8 and 11.2, are shown in the curves given in Figs. 1,
2, and 3. The shifting of the peaks with the change of pH shows a sufficient
agreement with previous experience (7–9) to indicate that they are β-

hydroxypyridines and not α- or γ-hydroxypyridines. To satisfy this requirement, barring most unlikely rearrangements during the dehydrogenation process, the hydroxyl group is therefore on carbon atom 23 of the steroid side chain. A substantiation of the β-hydroxypyridine character was obtained in the color reactions produced with the Folin-Denis reagent. As shown ! Kuhn and Wendt (7), a positive reaction is given only by β-
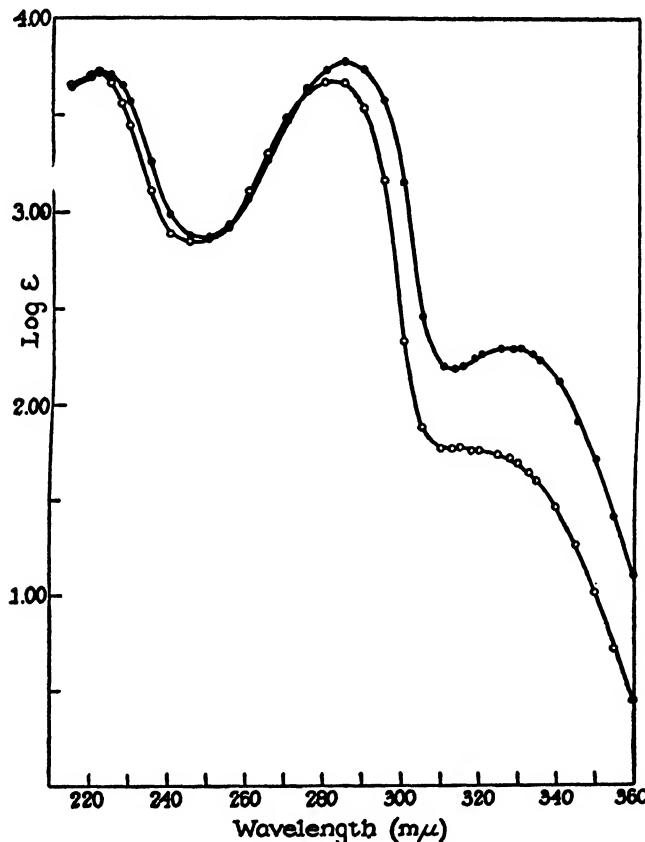
FIG. 1. O = C_8H_7ON from veratramine; ● = $C_8H_{11}ON$ from jervine; in methanol.

hydroxypyridines and not by α or γ derivatives. The blue color obtained with the C_8H_7ON base from veratramine was of a lighter shade than that from the jervine product, $C_8H_{11}ON$, and this in turn was less intense than the deep color given by pyridoxine, which was used for comparison. An attempt is in progress to synthesize these bases for substantiation of Formulas IV and V.

The investigation of the residue which remained undistilled in the de-

hydrogenation of veratramine is still in a preliminary stage.   However, the neutral hydrocarbon fraction, which was separated through alumina, has readily yielded in the first fractions a crystalline hydrocarbon (m.p., 153.5–155.5°).   This was indistinguishable in properties from the hydrocarbon $C_{22}H_{20}$ (m.p., 154–155°) previously obtained from jervine (2).   Like the jervine hydrocarbon, it failed to yield a stable picrate and gives a strong
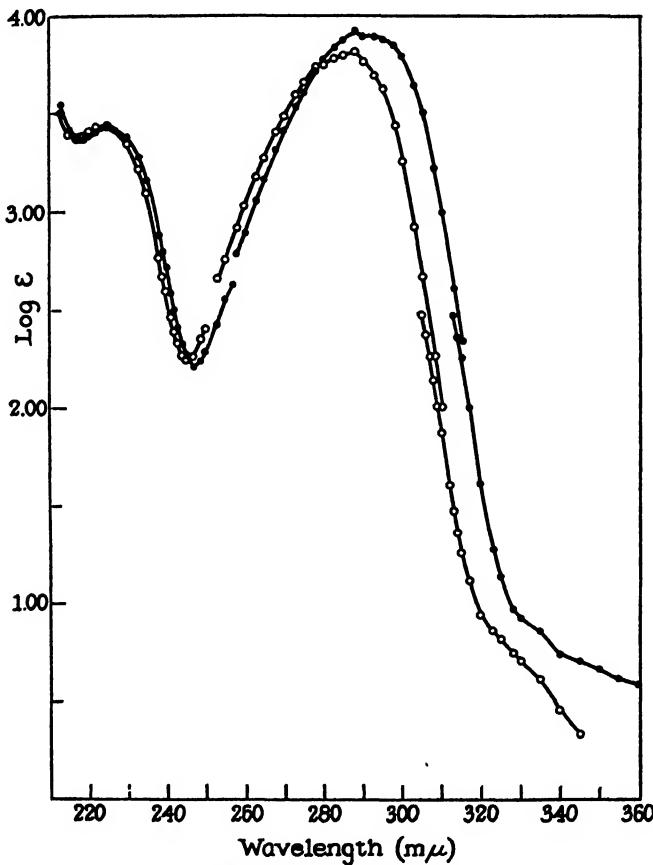
Fig. 2. $O = C_9H_7ON$; $\bullet = C_9H_{11}ON$; both in aqueous HCl of pH 1.85

test with tetranitromethane.   The absorption spectrum shown in Fig. 4 is also in essential agreement with that previously found with the jervine product.   In the earlier work a resemblance to the absorption curve of 1,2-benzofluorene was discussed.   However, the curve obtained is not incompatible with that to be expected of a chrysene homologue and this would be more consistent with recent data regarding the structure of jervine.   It is now planned to complete the study of these hydrocarbons.

From the data obtained a possible interpretation of the structure of veratramine is presented in Formula I. The close similarity of the ultraviolet absorption curves of veratramine and dihydroveratramine (4) already published shows that the reactive double bond is separate and not conjugated with those of the benzenoid ring. A recent determination in chloroform solution has shown for veratramine a rotation of $[\alpha]_D^{27} = -71.8°$ (c =

FIG. 3. O = C_6H_7ON; ● = $C_6H_{11}ON$; both in aqueous NaOH of pH 11.2

0.99) and for dihydroveratramine, $[\alpha]_D^{27} = +27.4°$ (c = 0.95). The molecular rotation difference from this of $-407°$ to be assigned to the double bond has not been interpreted from the available data. We are dealing with a benzenoid derivative. The Δ of $-298°$ shown by Barton and Klyne (10) for the Δ^5 bond is the nearest approach to the above difference. If the usual 3(β)-hydroxy-Δ^5-stenol character is to be assigned to veratramine, it would require that Ring C is benzenoid. However, it appears doubtful

that a Δ^5 double bond could resist a shift to Δ^6 for conjugation with such a near-by benzenoid Ring C.   If Ring B is assumed to be benzenoid, it is similarly true that a double bond at Δ^{15} should readily shift to Δ^{14} to conjugate with Ring B.   However, with Δ^{16} or Δ^{17} [20] there might be less likelihood of double bond shift.   But as a possible objection the molecular rotation contributions of such double bonds as listed by Barton and Klyne (10) and Fieser and Fieser (11) in otherwise saturated substances are $+31°$
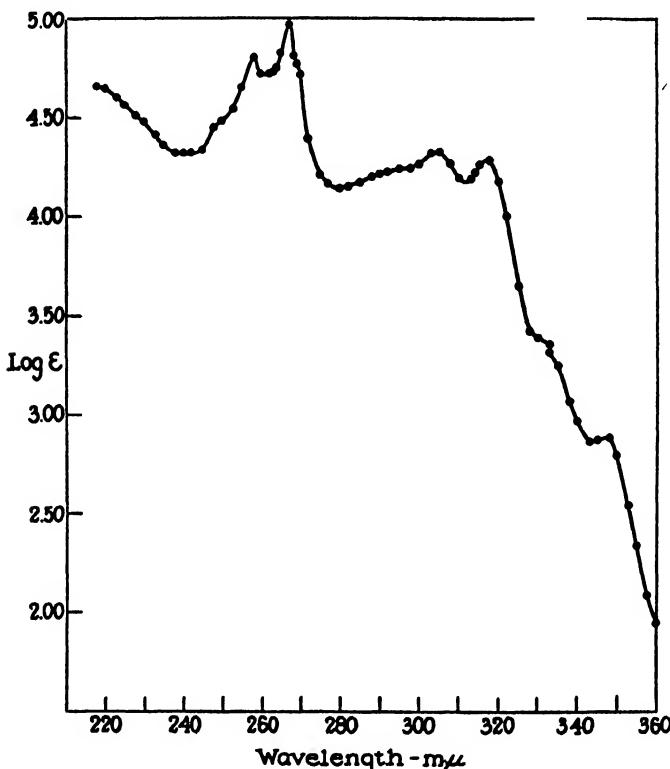
FIG. 4.   ● = $C_{22}H_{30}$ hydrocarbon

for Δ^{16} and $+35°$ for Δ^{17} [20].   If not in the side chain, a further possibility is a Δ^5 base with Ring D enlarged and benzenoid.

In the case of jervine, with one place of attachment of the oxidic linkage probably established on C^{23}, there remains in question its other point of attachment.   In the formation of isojervine it appears from its resistance to hydrogenation that a benzenoid structure has developed, even though the presence of the latter is not apparent in its ultraviolet absorption spectrum and is possibly masked by other contributing factors.   For

further information a preliminary study was made of its behavior on selenium dehydrogenation. However, the instability of isojervine in a different sense resulted in a large amount of apparent resinification. From the volatile basic material a small phenolic basic fraction was separated, which in turn yielded only a very small amount of the methylethylhydroxypyridine originally obtained from jervine. There was no 'evidence of the formation of the hydroxypicoline characteristic of veratramine. In view of the negative outcome of other studies of isojervine, its correct interpretation has not been completed. There remains the possibility that it retains the carbonyl group and that the isomerization involves removal of the 3-hydroxyl group, with double bond formation, and production of a benzenoid structure of Ring B, with accompanying rearrangement of the angular methyl group. Simultaneously, the two acylatable hydroxyl groups of isojervine may arise from cleavage of the oxidic bridge which may be more labile in jervine than in its hydrogenation products. Such structures would be represented by Formulas II and III for jervine and isojervine respectively.

This possibility is supported by the previously reported experience with the hydrolysis of pseudojervine (5). With 2 per cent HCl, cleavage occurred, with the production of a large fraction of isojervine and no jervine. Under similar conditions jervine itself was converted to isojervine to the extent of less than one-third, and the major part was recovered unchanged. This might suggest that during hydrolysis of the glycoside the liberated 3-hydroxyl group is activated and participates more readily in the double bond formation. There is some analogy for the loss of this hydroxyl in the conversion of periplogenin into trianhydroperiplogenin (12).

In the case of jervine the possibility of an oxygen bridge between carbon atoms 23 and 21 or between carbons 23 and 27 must be considered. But the production of 3-methyl-6-ethylpyridine and its 5-hydroxy derivative on dehydrogenation and the failure to detect any bases with oxygen attached to the side chains, as in the case of cevine (13), would appear to be against such an arrangement. An oxygen bridge to carbon 26 appears to be definitely excluded by the character of isojervine and its stability to acids.

<div align="center">EXPERIMENTAL</div>

Dehydrogenation of Veratramine—A ground mixture of 5 gm. of veratramine and 15 gm. of selenium contained in a flask, through which a very slow stream of nitrogen passed, was placed in a bath at 240°. The temperature during 20 minutes was gradually raised to 280° to permit water to distil off. During the following 30 minutes the temperature reached 300°. From the molten mass there was considerable gas evolution. Aside from the small amount, apparently water, very little condensed at first. After

20 minutes the temperature was gradually raised to 330–335° and maintained at this point for 2¼ hours. Gas evolution had subsided and a small amount of colored material had collected in the chilled receiver, although a less volatile and little colored product had accumulated in the upper portion of the flask. The distillate was worked up separately from the condensation product. After removal of some colored distillate with ether from the side arm of the flask, the washings were added to the few drops of distillate in the receiver. This extract (Fraction A) will be discussed below. The less volatile condensate (Fraction B) in the reaction flask above the solidified melt was carefully dissolved with ether, contact with the lower solid being avoided. The yellow ether solution was filtered from selenium and shaken with 2 cc. of 10 per cent HCl. The aqueous phase became a deep red, with precipitation of colored resinous salts. This was followed by a second 2 cc. of HCl and then repeatedly with small portions of water, which finally redissolved practically all of the solid. The remaining ether solution, which possibly contained some hydrocarbons and other neutral material, was not studied further.

The HCl solution was treated with excess 25 per cent NaOH until strongly alkaline, with accompanying color change to yellow. The turbid mixture, which smelled of pyridine homologues, was repeatedly extracted with ether and this extract was joined with a similar fraction to be described later in connection with Fraction A. The alkaline aqueous phase was saturated with CO_2 and then repeatedly extracted with ether. The dried extract yielded 0.125 gm. of a slightly colored oil, which crystallized from a small volume of ether as compact aggregates of wedge-shaped micro platelets. The yield of this fraction was 40 mg., and an additional 10 mg. were obtained from the mother liquor. After recrystallization from benzene it melted at 137.5–139° (corrected). When mixed with the $C_8H_{11}ON$ base from jervine previously described (2) (m.p. 145–147°), a marked depression was obtained. It was easily soluble in water, methanol, and ethanol.

C_6H_7ON.   Calculated.   C 66.02, H 6.47
              Found. (a)   " 66.40, " 6.72
                     (b)   " 65.96, " 6.53

The aqueous solution gave a brown-orange color with ferric chloride and coupled in alkaline solution with diazotized sulfanilic acid to a brown-yellow solution. This changed to a bright yellow on acidification, but returned to the former color with alkali. No difference was noted in parallel reactions with the substance from jervine.

The mother liquors of this hydroxypicoline contained much oily material, which was not studied further.

The above ether solution of Fraction A obtained from the reaction distil-

late was similarly treated to separate the basic phenolic fraction. The latter amounted to 60 mg. of resinous material. From the concentrated ether solution 5 mg. of crystalline phenolic base, identical with the above hydroxypicoline, were obtained.

The ether solutions of non-phenolic bases from Fractions A and B were joined and on concentration yielded a thick, dark brown oil with an odor suggestive of the higher pyridines. When the material was treated again with ether, some undissolved substance remained, and the filtrate on concentration gave about 50 mg. of residue. When dissolved in alcohol and treated with 50 mg. of picric acid in alcohol, a dark tar precipitated from which no crystalline material could be obtained. The supernatant solution yielded on concentration a small amount of tar mixed with crystals, which were collected with alcohol. On recrystallization from acetone, 4 mg. of light yellow needles were obtained which melted at 224–227°. This substance has not been identified.

<center>Found, C 44.09, H 3.60</center>

The attempt to obtain methylethylpyridine from this fraction was unsuccessful.

The undistilled residue which remained in the original dehydrogenation flask was extracted with benzene. The filtered dark brown solution was extracted with 5 cc. of 10 per cent NaOH. An appreciable amount appeared to dissolve, with some slight tar formation. This was followed by repeated water extraction. The alkaline aqueous extract was saturated with CO_2, which caused some precipitation and foaming, and then repeatedly extracted with ether. This yielded 0.15 gm. of a colored resin. The latter was not completely studied beyond the isolation of a small amount of hydroxypicoline.

The remaining benzene phase was shaken with 5 cc. of 10 per cent HCl, which caused precipitation of a dark red tar of salts. The acid extract was drained off and the mixture was again shaken with 5 cc. of 10 per cent HCl. This was followed progressively with 5 cc. portions of water until the latter became but little colored. However, most of the red tar of salts remained undissolved on the funnel walls and the benzene phase was separated, followed by rinsings. The HCl salt fraction was dissolved in methanol and the red solution was cleared from suspended material by centrifugation. The concentrated solution when made alkaline changed to a brown color. Extraction with benzene yielded 1.15 gm. of a colored resin of bases. This material in acetone yielded 0.17 gm. of a crystalline product, m.p. 248–250°, which appears to be a phenolic base and of the general order of cevanthridine. A report on this substance will be presented in a later paper.

The dried benzene solution, which contained essentially neutral material,

yielded 2.23 gm. of a dark colored tar. A solution of this in 15 cc. of benzene was chromatographed through 60 gm. of Al_2O_3 and developed with benzene. The color spread down in bands and the material quickly emerged as a yellow solution. At this point the first 15 cc. yielded 0.15 gm., which did not crystallize. The following 10 cc. contained 0.6 gm. of a partly crystalline resin. The third 10 cc. gave 0.29 gm., which crystallized more copiously, and the fourth 10 cc. gave 0.11 gm. of partly crystalline material. The following 10 cc. fractions eluted progressively less material, which from the tenth to the eighteenth fractions remained almost constant at about 20 mg. and again gradually crystallized. More material was finally obtained in fractions with 1 per cent methanol in benzene. The study of these later fractions has been postponed.

The second fraction was crystallized from a small volume of benzene at 0° and collected in the cold with a little toluene. 72 mg. were obtained. The third and fourth fractions yielded 45 and 28 mg., respectively. Further study showed them to be essentially identical. After repeated recrystallization from benzene-alcohol and then twice from ether, the substance formed long diamond-shaped leaflets which melted at 153.5–155.5°. The mixture with the $C_{22}H_{20}$ hydrocarbon from jervine (2) sintered at 151° and melted at 153.5–155.5.° The jervine product taken again for comparison sintered at 152° and melted at 153.5–155.5°. The ultraviolet absorption spectrum is shown in Fig. 4.

$C_{22}H_{20}$. Calculated.   C 92.91, H 7.09, mol. wt. 284.18
   Found. (a)   " 92.55, " 7.27   "   " 274, 267.3
     (b)   " 92.82, " 6.89

Although concentration of a solution of the hydrocarbon and picric acid in acetone gave a red color, only leaflets of the hydrocarbon crystallized. When collected in three successive fractions, practically all of the hydrocarbon was recovered. Similarly, from benzene no picrate was obtained. With trinitrobenzene there appeared to be little tendency to form a stable compound.

Dehydrogenation of Isojervine—A mixture of 5 gm. of isojervine and 15 gm. of selenium was heated as in the previous case at first at 240°, and then the temperature was gradually raised to 325–330° and held at this point for 2 hours. At about 310° an unusual amount of almost black material filled the upper portion of the apparatus and considerable dark oil accumulated in the receiver. When the distillate was dissolved in ether, the basic fraction was extracted with HCl. From this solution the bases were liberated with an excess of NaOH and reextracted with ether. The mixture smelled distinctly of pyridine bases and was not studied further. The NaOH solution, after neutralization with CO_2, was in turn extracted with ether. From this only 15 mg. of a red-brown resin were obtained. The solution in

ether, when cleared with norit and concentrated, yielded characteristic, almost rectangular leaflets. 3 mg. were collected with ether. The substance melted at 142–143° and, when mixed with the jervine product (m.p. 145–147°), there was no appreciable depression. When mixed with the hydroxypicoline from veratramine, a marked depression was obtained.

$C_8H_{11}ON$. Calculated. C 70.02, H 8.08; found, C 69.75, H 7.89

Investigation of the undistilled residue in the reaction flask for additional phenolic basic material gave only about 15 mg. of colored resin, from which none of the $C_8H_{11}ON$ compound could be crystallized.

No attempt has been made to investigate the other fractions of the dehydrogenation.

All of the analytical data have been furnished by Mr. D. Rigakos of this laboratory.

SUMMARY

The dehydrogenation of veratramine has yielded a phenolic base, C_6H_7ON, which is closely related to the phenolic base, $C_8H_{11}ON$, previously obtained from jervine. The change in the ultraviolet absorption spectra of these substances with change of pH, as well as a positive color reaction, given with the Folin-Denis reagent, affords the strongest evidence that they are β-hydroxypyridine bases, and that the former is 3-methyl-5-hydroxypyridine and the latter 3-methyl-6-ethyl-5-hydroxypyridine. The production of these substances in conjunction with other data has permitted certain conclusions regarding the structure of the parent steroidal bases.

The hydrocarbon $C_{22}H_{20}$, previously obtained from jervine, has also been found with other crystalline substances among the dehydrogenation products of veratramine.

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PURITY STUDIES ON POLYPEPTIDE ANTIBIOTICS: BACITRACIN*

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As part of a broader study on Counter-current Distribution, the question of the purity of available polypeptide preparations has been taken up. We have found it possible to fractionate gramicidin, tyrocidine, gramicidin-S, and bacitracin samples into several different polypeptide components. In each case, except with the tyrocidine fractions which have not been studied antibiotically as yet, a variation in activity with the different components has been noted. They differ also in the amino acids which they contain as well as in the proportion of these amino acids.

Bacitracin for the most part has been furnished by the Commercial Solvents Co. We are indebted to them and to Miss Johnson and Dr. Meleney for the bioassays. Bacitracin has not proved to be the most ideal type of substance to distribute because of its tendency to give skewed curves and because of its lack of stability. In attempts to distribute it in neutral 2-butanol/water, the curves obtained were typical transformation curves. 2-Butanol/water acidified with acetic acid was much more satisfactory and showed only slight transformation during a run. A typical result is shown in Figure 1. This was made with a sample of 46 unit material. A calculated curve superimposed on the weight curve showed the latter to be somewhat skewed. An activity curve also was skewed but seemed to indicate a single active substance being present in the major component. A considerable band of low activity occurred to the right. Absorption spectrum measurement indicated mixtures in tubes 40 to 60.

An interesting feature of this distribution is that a maximum activity of 66 units was indicated. This was on a dry-weight basis when dried at 100° in vacuum. Material recovered from the main band by freeze-drying was a white, highly hygroscopic powder which had an activity slightly less than the maximum obtained from the curve. That it had lost a certain amount of activity through denaturation on isolation was confirmed by redistribution. In this case a small percentage of material of low activity appeared on the left of the main band.

Hydrolysis in 6 N hydrochloric acid followed by paper chromatography gave spots corresponding to phenylalanine, leucine, isoleucine, cystine, valine, his-

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tidine, ornithine, lysine, and glutamic and aspartic acids. Strong evidence is thus furnished that the active principle is a polypeptide of considerable size.

At about this stage of our work certain toxic manifestations were c ount red by others on the clinical side and it became desirable to have more c ful chemical investigations in order to see if some closely related toxic substance could be removed by fractionation. This problem can be approached in two ways, namely, higher numbers of transfers or a change to a more specific system. We have investigated both approaches.

An all-glass apparatus of simple construction has been devised which is shown in Figure 2. This particular one contains 108 units but it would appear
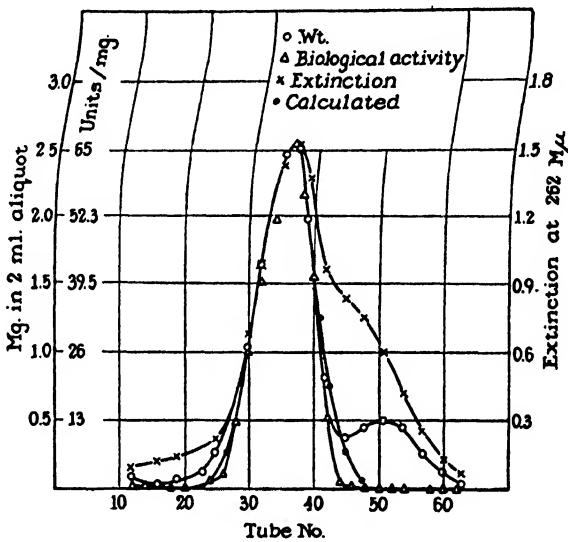
FIG. 1. 85 transfer distribution of bacitracin in 2-butanol/3% acetic acid.

that this number could be extended several fold if necessary for a specific problem. Enlarging is merely a matter of adding individual units. An interesting feature is that strong acids such as hydrochloric acid can be used.

A run on bacitracin using a system made with methanol, 0.1 N hydrochloric acid and chloroform gave the pattern shown in Figure 3. The overall aspects are not too different from those with the first system. Inactive material is shown at fractions 140 to 120 and a certain amount of foreign material is demonstrated on the left. Somewhat more inactivation was encountered during the run than with the acetic acid system. When inactivation in acid solution occurs, the transformation products appear on the left. When inactivation in neutral or alkaline solution occurs, the products appear on the right. A hydrochloride with rather nice properties was recovered from the peak tubes.

FIG. 2. Glass counter-current distribution apparatus containing 108 tubes.
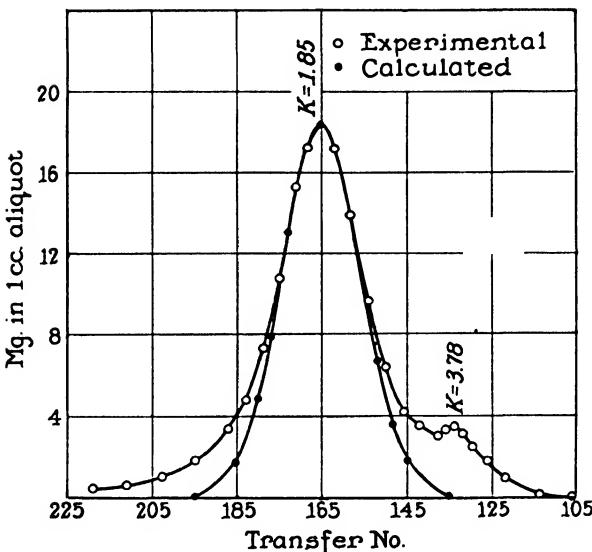
FIG. 3. Distribution of bacitracin in a system containing chloroform, methanol and 0.1 N hydrochloric acid.
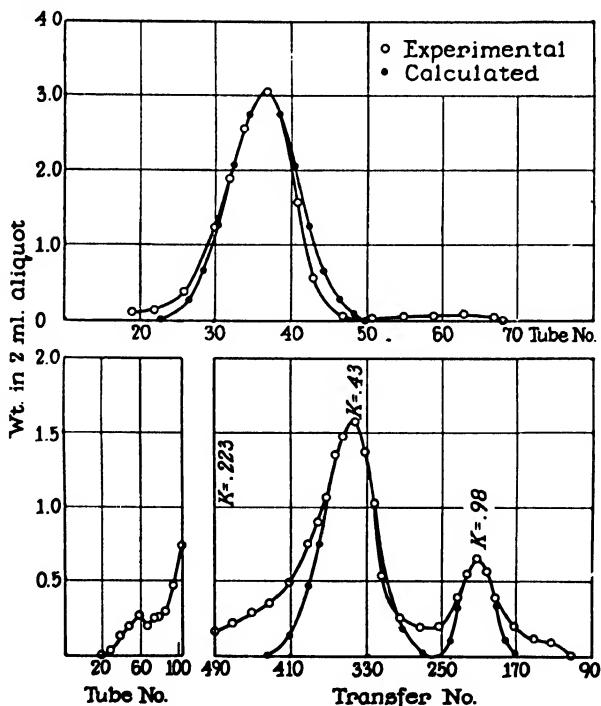
FIG. 4. Comparative distributions of bacitracin in 2-butanol/3% acetic acid.

FIG. 5. Appearance of purified bacitracin.
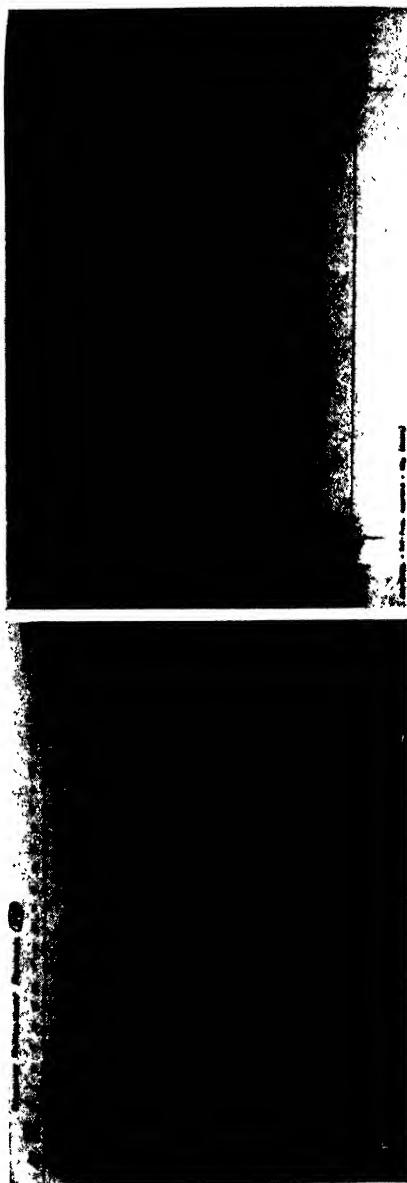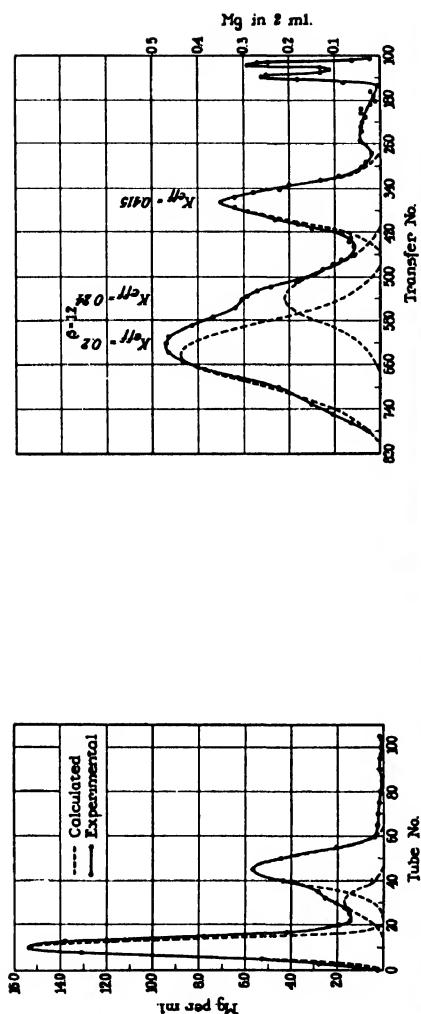
FIG. 6. Distribution of the hydrolysis products of bacitracin.

At least one sample of bacitracin was not entirely stable in the solid state. This sample originally had an activity of 55 units and gave quite a good pattern, Figure 4, upper pattern, with 75 transfers and the acetic acid system. Only a small amount of inactive material on the right and left was obtained. After standing in the cold room for about nine months, the lower pattern was obtained with the same system but with the application of 490 transfers. The activity of the sample after nine months was 47 units. The fractions were recovered as usual by freeze-drying and, to our surprise and gratification, material recovered from the main band on one run had definite organization as shown in Figure 5. None of the fractions recovered from other regions showed this organization. Furthermore, the recovered material had an activity of 60 units without further drying.

It would now appear fairly certain that this material is either a single substance or a mixture of very closely related substances. The evidence is not as rigorous as we would like because of inherent instability but at least the experience in two different systems points toward something definite on which to base future work.

Hydrolysis in boiling 6 N hydrochloric acid for 24 hours, evaporation of the excess hydrochloric acid, and distribution of the mixed hydrochlorides in a 2-butanol/ammonium acetate system has given the pattern shown in Figure 6. The bands from right to left are a dipeptide containing phenylalanine and isoleucine; peptide material containing phenylalanine, isoleucine, and apparently ornithine; phenylalanine; leucine; isoleucine; ammonium chloride; histidine; and, finally, a mixed band containing cystine, lysine, and aspartic and glutamic acids. Redistribution in other systems has permitted further resolution of the mixed band. Spotting of every tenth fraction on a broad paper chromatogram permits ready control of the separation.

The original "valine" spot has been isolated in crystalline form from tubes 60–70 but it is different from valine. It appears to be absent from the most highly purified material. All the spots indicated by paper chromatography have been isolated in crystalline form with the correct carbon and hydrogen analysis except lysine and ornithine. In addition, peptides have been isolated. The amino acids isolated were of the *l-*, *d,l-*, and *d-*configurations, as follows: *l*-histidine, partially racemic *l*-leucine, *l*-cystine and *l*-glutamic acid, *d,l*-phenylalanine, *d,l*-aspartic acid, and partially racemic *d*-isoleucine.

THE EFFECT OF CENTRIFUGAL FORCE ON GALVANIC POTENTIALS: (A) THE TRANSFERENCE NUMBERS OF POTASSIUM IODIDE, (B) THE IODIDE-IODINE ION

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The effect of gradients of centrifugal force on the potentials of simple galvanic cells has been studied by Des Coudres[1] and more extensively by Tolman,[2] and some preliminary measurements have been reported by MacInnes.[3] Tolman and MacInnes used galvanic cells of the type

$$(Pt); I_2, KI, I_2; (Pt) \qquad\qquad (A)$$
$$r_2 \qquad\qquad r_1$$

in which two, otherwise identical, iodide-iodine electrodes with a uniform solution between them are placed at radii r_1 and r_2 in a centrifugal field. These researches have been interpreted by the equation

$$EF = 2\pi^2 n^2 (r^2 - r_1^2)[t_K(M_{KI} - \bar{V}_{KI}\rho) - (M_I - \bar{V}_I\rho)] \qquad (1)$$

in which F is the faraday, n is the number of revolutions per second, t_K is the transference number of the cation constituent, ρ is the density of the solution, M_{KI} and $\bar{V}_{KI}$ are the molecular weights and partial molal volumes of potassium iodide, and M_I and $\bar{V}_I$ are the atomic weight and partial atomic volume of iodine. It will be shown below that this is a limiting form of an equation which is based on more complete knowledge of the mechanism of cell A.

Studies of the closely related effect of differences of height on the potentials of galvanic cells have been made by Des Coudres[4] and more recently by Grinnell and Koenig.[5]

The results to be described below are the outcome of a long research undertaken to develop the centrifugal e. m. f. procedure into a precision method for obtaining transference numbers. It is of particular importance in that it can be

(1) Des Coudres, *Ann. Physik*, **49**, 234 (1893).

(2) Tolman, *Proc. Am. Acad. Arts Sci.*, **46**, 109 (1910); This Journal, **33**, 121 (1911).

(3) MacInnes, *Ann. New York Acad. Sci.*, **43**, 243 (1942).

(4) Des Coudres, *Ann. Physik*, **57**, 232 (1896).

(5) Grinnell and Koenig, This Journal, **64**, 682 (1942).

used, as has been demonstrated by experiments already made, in the mination of transference numbers in non-aqueous solvents, where, due to Joule heat, the Hittorf and moving boundary methods encounter difficulties.

The Apparatus

The apparatus,[6] and the electrical connections, are shown diagrammatically in Fig. 1. The rotor R-R, which is a disk of magnesium 23 cm. in diameter and 5 cm. thick, is turned in a horizontal plane by means of the pressure of the disk D on the plate P, which is rotated by the synchronous motor M. The rotor speed can be varied
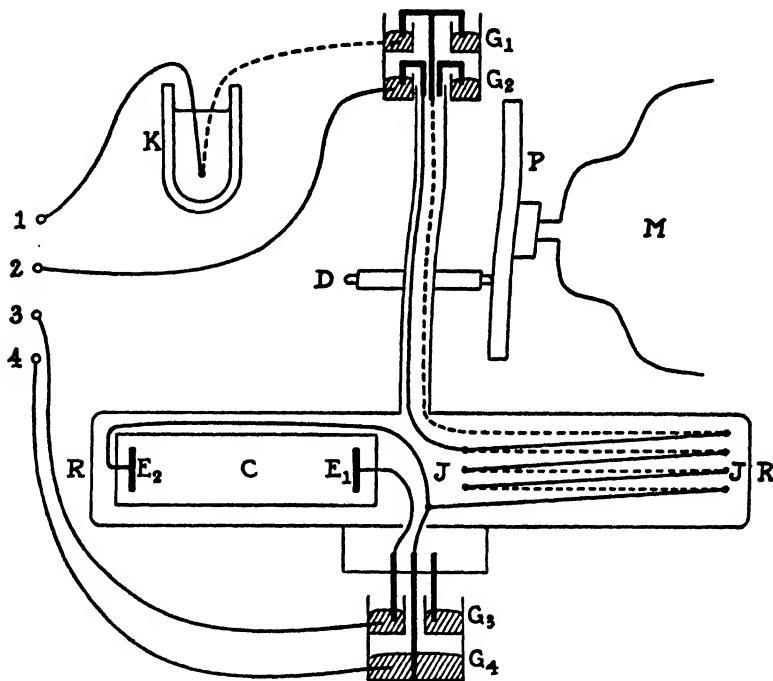
FIG. 1. Diagram of apparatus.

by changing the position of the disk D with the relation to the plate P. The potential between the electrodes E_1 and E_2 of the galvanic cell C can be measured, during the rotation, by electrical contacts through the mercury commutators G_3 and G_4. The difference of temperature at radii corresponding to the positions of E_1 and E_2 is obtained by means of the thermojunctions J-J. Twenty-two junctions are used, and are actually arranged around cell C. The resulting thermopotential is measured between the commutators G_3 and G_4. Finally, the temperature of the rotor is found using a single junction in the rotor, the commutators G_1 and G_2 and the reference junction

(6) The apparatus is more fully described in an article by Ray and MacInnes in the *Rev. Sci. Inst.*, **20**, 52 (1949).

in the external ice-bath K. Thus by shifting the leads of the potentiometer to the appropriate pairs of the contacts 1, 2, 3 and 4 the e. m. f. of the cell, the differential temperature, and the temperature of the rotor may be measured. The temperature measurements are essential since the measured potentials have been found to be considerably aff ted by radial temperature gradients. Such gradients are minimized by surroundin͆ ͵e rotor with a chamber in which a vacuum of 1 μ or better can be main-
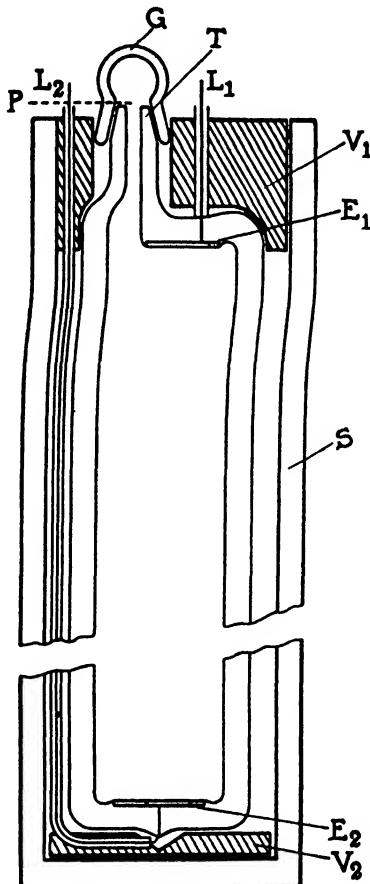
FIG. 2. The galvanic cell.

tained. This prevents the production of appreciable heat through gas friction. The heat generated at the vacuum bearing is controlled by the circulation of cooling water. Radial temperature differences are reduced to less than 0.01° by empirically choosing the temperature of the water. This arrangement also greatly reduces the rise, during a determination, of the temperature of the rotor.

The cell used in obtaining the data to be described below is shown in Fig. 2. The electrodes E_1 and E_2, which are platinum disks 1 cm. in diameter and 6.4 cm. apart,

are sealed into the Jena 16 III glass ends of the cell. The platinum leads, L_1 and L_2, are enclosed in flexible plastic tubing. Filling and removal of the solution are carried out through the tube T which is closed with a ground-glass cap G. The cell is enclosed in the brass shell S, the space between the shell and the cell being filled with vaseline. This semi-fluid material helps to equalize the pressure on the two sides and bottom of the cell when in a centrifugal field. The Bakelite spacers, V_1 and V_2, hold the glass cell in a fixed position in the shell S.

To determine the radii r_1 and r_2 of eq. 1 the procedure was as follows. The glass portion of the cell shown in Fig. 2 was filled with, and immersed in, a microscope immersion oil with the same coefficient as glass, and the distances of the electrodes E_1 and E_2 from the top edge of the cell, P, which was ground flat, were read with an accurate comparator, equipped with a travelling microscope. The cell was then assembled, as shown in the figure, inserted in the centrifuge, and run at top speed to ensure definite settling into place. The cell assembly was then placed on a surface plate and the distance between the top edge P and the bottom of the brass shell S was measured with a depth gage. When placed in the rotor R-R of Fig. 1, the cell and its counterpoise rest against disks closing the ends of the channel. The effective diameter of the rotor, *i.e.*, the distance between these disks, was measured with the aid of a precision vernier caliper. With these data the radii r_1 and r_2 may be computed. Since the electrodes E_1 and E_2 are not exactly parallel the factor $(r_2^2 - r_1^2)$, which for the cell used in these experiments was 86.04 cm.,[2] may be in error by about $\pm 0.2\%$. Another design of cell which allows for greater precision in this factor has also been used in our work.[6]

It is quite important that the counterpoise have the same moment of inertia as the cell, *i.e.*, imitate it closely in distribution of weight, since otherwise the rotor may precess, and may not rotate about its geometrical center.

The mercury commutators G_1, G_2, G_3 and G_4 are described in detail elsewhere.[6] With them it was possible to make measurements to one microvolt, or better, of potentials developed in the rotor, this precision being necessary because the highest potentials determined are of the order of one millivolt.

The measurements of the speeds of rotation, which ranged between 400 and 7200 r. p. m., were made with the aid of stroboscopic patterns, as described in a paper from this Laboratory.[7] The rotating top surface of the commutator G_1 of Fig. 1 is painted black, with a white radial streak. This surface is illuminated by a stroboscopic lamp, which is operated from the local a. c. current, and yields flashes at the rate of 60 per second. At definite speeds stationary stroboscopic patterns are observed which are related by the formula r. p. m. $= (3600 \times m)/n$ in which n is the number of bands in the pattern, and m is the "multiplicity." Patterns are observed for integral values of m and n except when they have a common factor. As the same pattern occurs at a series of related speeds it is necessary to have a rough preliminary estimate of its value. This is furnished by a scale attached to the adjustment mechanism of the disk D of Fig. 1. Since stationary patterns could be obtained for indefinite periods, with occasional slight manual adjustments, the accuracy of the speed measurement was nearly that of the a. c. source, and was more than necessary for our purpose.

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(7) MacInnes, *Rev. Sci. Inst.*, **14**, 14 (1943).

The Preparation of the Solutions

The measurements described in this paper were made with 0.1941 N potassium iodide, to which varying amounts of iodine were added. In preparing this solution the best commercial salt was recrystallized several times, dried in an electric oven, followed by heating to 500° in a platinum boat in a current of purified nitrogen. The material was then weighed with the aid of the Richards bottling apparatus[8] procedure, after which the salt was dissolved in a weighed amount of conductivity water. A solution 0.1941 N in potassium iodide and 0.1585 N in iodine was made by adding a weighed amount of iodine (several times resublimed) to the stock potassium iodide solution. The solutions with smaller proportions of iodine were made by diluting, by volume, with carefully calibrated pipets, with the stock potassium iodide solution.

After many experiments designed to discover the source of persistent irregularities in the measurements, a source of difficulty was located in the minute suspended particles in the solutions. These were present although every effort had been made to ensure cleanliness in preparing the solutions. Such particles are far more important in this centrifugal work than in other e. m. f. measurements, since particles that are denser than the solution in which they are suspended will drift toward the outer, and less dense ones to the inner electrode, in both cases producing contamination of the platinum surface, and thus affecting the measured potential. To overcome this difficulty many of the solutions were ultrafiltered, with suction, through collodion membranes supported on sintered glass. This procedure yielded solutions which appeared optically clear in a Tyndall beam. Another procedure gave somewhat less complete, but apparently sufficient, removal of suspended material. This was the repeated filtration between each crystallization of the hot saturated solutions of potassium iodide, using the most dense hardened filter paper obtainable. The paper was pre-washed and supported on a steam-heated funnel. Solutions made up from this salt were very nearly optically clear and were used for part of the work. In earlier work the results appeared to be influenced by the presence of oxygen in the solutions, and elaborate precautions were made to exclude it from the solutions and the surroundings of the cell. With the use of ultrafiltration, the need of these precautions apparently disappeared. It seems possible that a really clean surface of platinum can overcome the disturbing effect of a small concentration of oxygen, whereas a contaminated surface is unable to do so. However, we do not consider the matter to be fully settled.

THE EXPERIMENTAL RESULTS

The Effects of Temperature Control.—As mentioned above, correct, constant values of the e. m. f. of the galvanic cell were obtained only when there was no difference of temperature between the radii of the rotor corresponding to the positions of the electrodes E_1 and E_2. This is clearly indicated by some typical measurements illustrated in Fig. 3. In this figure the top curve shows the potentials of the galvanic cell, the next lower one the temperature of the rotor, and the bottom curve the difference of temperature at the points occupied by the electrodes, all plotted as functions of the time, the zero being the mo-

(8) Richards and Parker, *Proc. Am. Acad. Arts Sci.*, **32**, 59 (1896).

ment at which the rotor attained a speed of 5400 r. p. m. It will be seen that there is a quick rise of the e. m. f. of the cell, followed by a slower decrease. During the same period there is a slow increase of the temperature of the rotor, and a corresponding rise to a low maximum of the differential temperature. At a time indicated by the vertical dotted line running water was used to fix, at an appropriate point, the temperature of the rotor bearing. It will be observed in the figure that the differential temperature dropped rapidly to nearly zero, and shortly after the e. m. f. of the cell, and the temperature of the rotor, assumed constant values.

With experience, it was possible to adjust the temperature of the bearing at the start so as to avoid appreciable temperature gradients in the cell during the
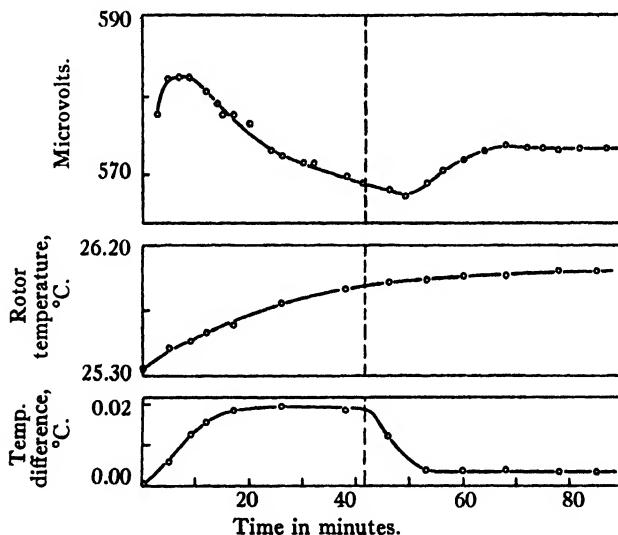
Fig. 3. Effect of temperature difference on e. m. f. of cell and on rotor temperature.

measurements. The measured potentials of the cell at each speed were, in general, established immediately, and remained constant, within one or two microvolts, for the period of the measurement, generally about ten minutes.

A Typical Experiment.—The results of a typical experiment are shown in line A of Fig. 4, in which the values of the potentials E, in microvolts, are plotted as functions of the square, n^2 of the speed of rotation in seconds. According to eq. 1 and the following eq. 8 this relation should be linear and pass through the origin. That A is a straight line is shown by plotting the slopes, E/n^2, against n^2 as shown in line B. The slope is thus seen to be constant within a very small limit of error. These data were obtained using solution 8, of Table I, in the galvanic cell.

If, at the higher speeds, the experiment is prolonged the measured potentials drop slowly. This is to be expected, since, under the influence of the centrifugal force, there is a tendency of the salt and of the iodine to drift in the direction of the outer electrode. In an ultracentrifuge at much higher speeds than those used in our experiments Pedersen[9] attained sedimentation equilibrium for a number of salts, such as cesium chloride and lithium iodate.

The e. m. f. data plotted in Fig. 4 and recorded in Table I were obtained using a series of increasing speeds. The same accuracy has not yet been ob-
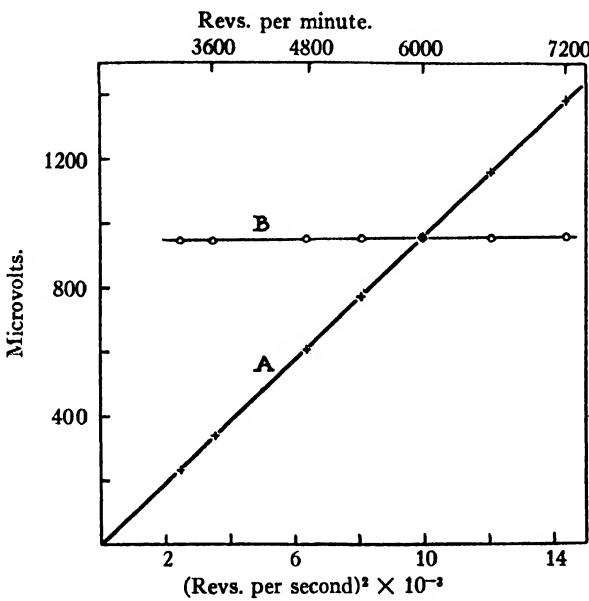
FIG. 4. Results of a typical experiment.

tained with decreasing speeds, a phenomenon for which we can give no explanation.

A Summary of the Measurements.—Table I contains the results of a series of measurements on solutions of potassium iodide containing 0.1941 mole per liter at 25° and in addition varying amounts of iodine. The normalities of the iodine, *i. e.*, gram atomic weights per liter, are shown in column 2 of the table. The densities of the solutions, given in column 3, were determined by a magnetic float method which will be described in a future publication from this Laboratory. In the fourth column are given the observed molar conductances, $\Lambda_m = 1000 \, \kappa/0.1941$, in which κ is the measured specific conductance. We are indebted to Dr. Theodore Shedlovsky for aid in making these measurements,

(9) K. O. Pedersen, *Z. physik. Chem.*, **A170**, 41 (1934).

which were made with the conductance bridge developed in this Laboratory, and in an oil thermostat regulating to 0.003°. The E/n^2 values given in column 5 were obtained by the method of least squares from the original data. Each "run" consisted of an experiment such as is shown in the results plotted in Fig. 4, with the potentials, E, measured at seven different speeds. For the cases in which more than one run was made, as indicated in column 6, the average deviation in per cent is given in the following column.

TABLE I

Results of Measurements of Potassium Iodide-Iodine Solutions

	Iodine normality	Density 25°	Molar cond. Λ_m 25°	$E/n^2 \times 10^3$ obs.	No. runs	Av. dev., %	$E/n^2 \times 10^3$ comp.
1	0	1.02037	126.70	(6.71)			6.68_4
2	0.00165	1.02053	126.57	6.73_8	2	0.1	6.71_3
3	.00990	1.02135	125.91	6.87_4	4	.1	6.85_3
4	.01981	1.02230	125.09	6.96_7	2	.05	7.00_6
5	.03961	1.02418	123.49	7.38_0	5	.4	7.34_1
6	.07922	1.02798	120.31	8.04_9	1		8.04_7
7	.1188	1.03178	117.15	8.85_1	4	.6	8.79_0
8	.1585	1.03558	114.05	9.58_8	1		9.60_4

Discussion of the Results

It will be seen from the data in Table I that the value of the quantity E/n^2 increases quite rapidly as the proportion of iodine in solution ascends. In the following discussion it is shown, quantitatively, that the effect is due to the effect of an iodide-iodine complex on the mechanism of the cell.

During the passage of current through the cell

$$(Pt); I_2, KI, I_3; (Pt) \tag{A}$$

the electrochemical reaction

$$I^- = I^0 + \epsilon^- \tag{2}$$

occurs at the anode and at the cathode. Here

$$\epsilon^- + I^0 = I^-$$
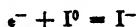
I^0 represents iodine in an uncharged condition. It may be in the form of I_2 or as a complex I^-_{j+1} with the iodine ion, in which j is the number of equivalents of uncharged iodine in the complex. During the passage of one faraday F through the cell, t_K equivalent of potassium ion constituent will migrate from the region of the anode and appear at the cathode, t representing a Hittorf transference number. The remainder of the current is transported in the reverse direction by t_I equivalent of I^- and t_C equivalent of the complex ion.

The net loss around the anode is t_K equivalent of potassium ion and $(1 - t_I - t_C) = t_K$ equivalent of negatively charged ion. Thus there is a net loss of t_K equivalent of potassium iodide from the region of the anode. Since reverse phenomena occur at the cathode, a transport of t_K equivalent of the salt from one electrode to the other takes place. This is accompanied by the appearance of one equivalent of I^0 at the anode by the electrochemical reaction, eq. 2, and jt_C equivalent of that material by transference.

The transport process per faraday for the galvanic cell A is therefore t_K equivalent of potassium iodide from the anode to cathode and $(1 + jt_C)$ equivalents of I^0 in the reverse direction. If gradients of chemical potential $\Delta\mu$ exist in the cell the Gibbs free energy ΔZ of its reversible operation will be

$$- \Delta Z = EF = t_K\Delta\mu_K - (1 + jt_C) \Delta\mu_{I^0} \tag{3}$$

Since the operation of the cell takes place in a centrifugal field the chemical potentials μ will be functions of the radius r in addition to the usual variables which are temperature, pressure, P, and the mole fractions of the components of the solution.

Assuming constant temperature and uniform concentration of the solution in the cell we have

$$\frac{d\mu_i}{dr} = \left(\frac{\partial\mu_i}{\partial r}\right)_P + \left(\frac{\partial\mu_i}{\partial P}\right)_r \frac{dP}{dr} \tag{4}$$

Now the change of μ_i with r is given by

$$(\partial\mu_i/\partial r) = - M_i\phi \tag{5}$$

in which ϕ is the centrifugal force per unit mass and M_i is the molecular or atomic weight. The negative sign is due to the fact that the energy of a component is increased by movement toward the center of rotation. The differential Gibbs free energy dZ of a solution is given by

$$dZ = -sdT + VdP + \mu_1 dN_1 + \mu_2 dN_2 \ldots \mu_i dN_i \tag{6}$$

in which $N_1, N_2, \cdots, N_i$ represent the numbers of moles. Since dZ is an exact differential the partial molal volume $\bar{V}_i$ can be obtained by the cross differentiation[10]

$$\partial\mu_i/\partial P = \partial V/\partial N_i = \bar{V}_i \tag{7}$$

Substituting $dP/dr = \rho\phi$ and eqs. 5 and 7 into eq. 4 we obtain

$$-d\mu_i/dr = \phi(M_i - \bar{V}_i\rho) = 4\pi^2 n^2 r(M_i - \bar{V}_i\rho) \tag{8}$$

(10) The thermodynamic function, Z, applicable to processes at constant temperature and pressure, is of service here since, although there is a gradient of pressure along the galvanic cell, the cell mechanism does not involve any alteration of this pressure distribution. Equations 6 and 7 are eqs. Nos. 92 and 272 of Gibbs' "Equilibrium of Heterogeneous Substances."

since $\phi = 4\pi^2 n^2 r$, in which n is the number of rotations per second. Using this expression in eq. 3 after integrating between radii r_2 and r_1 yields[11]

$$-EF = 2\pi^2 n^2(r_2{}^2 - r_1{}^2) \times [t_{\mathrm{K}}(M_{\mathrm{KI}} - \bar{V}_{\mathrm{KI}}\rho) - (1 + jt_{\mathrm{C}})(M_{\mathrm{I}} - \bar{V}_{\mathrm{I}}\rho)] \qquad (9)$$

It will be observed that this equation reduces to eq. 1, as the proportion of iodine, and thus the transference number t_{C}, decreases. Thus using eq. 1 and a limiting value of E/n^2 obtained by a short linear extrapolation from the data in Table I the transference number of the potassium ion in 0.1941 normal potassium iodide is found to be $t_{\mathrm{K}} = 0.487_3$. This agrees closely with the value 0.4887 obtained by Longsworth[12] by the quite independent method of moving
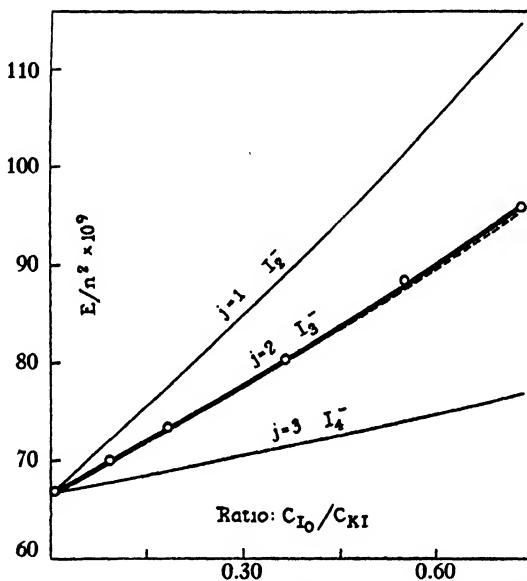
Fig. 5. The variation of E/n^2 values with the ratio $C_{\mathrm{I}}^0/C_{\mathrm{KI}}$.

boundaries. In our computation values of the partial molecular and atomic volumes $\bar{V}_{\mathrm{KI}}$, and $\bar{V}_{\mathrm{I}}$ are necessary. Longsworth gives a formula, based on existing density data, from which $\bar{V}_{\mathrm{KI}} = 46.34$ is obtained, and the value $\bar{V}_{\mathrm{I}} = 30.31$ was computed from the density data in Table I. These quantities will be the subject of another contribution from this Laboratory.

With the aid of the conductance measurements, given in Table I, for potassium iodide solutions, with varying proportions of added iodine, a test of the validity of eq. 9 may be made. Assuming Kohlrausch's law of independent

(11) This equation is equivalent to equation 83, for the effect of height, in the paper by Koenig and Grinnell, *J. Phys. Chem.*, **44**, 463 (1940).

(12) Longsworth, THIS JOURNAL, **57**, 1185 (1935).

ion mobilities[18] the measured equivalent conductance Λ_m is given by

$$\Lambda_m = \lambda_K + (1 - R/j)\lambda_I + (R/j)\lambda_C \tag{10}$$

in which the λ values are equivalent conductances of ion constituents; R is the ratio C_{I^0}/C_{KI}, and j is, once more, the number of equivalents of uncharged iodine carried by the complex. From this equation

$$\lambda_C = \lambda_I - (j/R)(\lambda_{KI} - \Lambda_m) \tag{11}$$

and also

$$t_K = \lambda_K/\Lambda_m \text{ and } t_C = R\lambda_C/j\Lambda_m \tag{12}$$

since transference numbers are the proportion of the total current carried by a given ion constituent. Substituting these values in eq. 9 we have

$$-FE = 4\pi^2 n^2 (r_2^2 - r_1^2)$$
$$\times \left[\frac{\lambda_K}{\Lambda_m} (M_{KI} - \bar{V}_{KI}\rho) - \left(1 + \frac{R\lambda_I - j(\lambda_{KI} - \Lambda_m)}{\Lambda_m}\right)(M_I - \bar{V}_I\rho) \right] \tag{13}$$

To test the validity of this equation in terms of our experimental results we must have, in addition to the data given in Table I, values of the ion conductances λ_K and λ_I. With the aid of Longsworth's[12] value 0.4887 of t_K for 0.2 N potassium iodide and the value of Λ_m for solution 1 of Table I, $\lambda_K = 61.92$ and $\lambda_I = 64.78$ are obtained.

On the assumption that for the complex I^-_{j+1}, j should be an integer, values of E/n^2 were computed for $j = 1, 2$ and 3, corresponding to ratios R from the data in Table I. Those for $j = 2$ are given in that table in the eighth column, and are seen to agree closely with the quantities E/n^2 obtained experimentally. That the experimental results indicate the presence of the complex I_3^-, and are not in accord with I_2^- or I_4^- is shown graphically in Fig. 5 in which values E/n for $j = 1, 2$ and 3 computed from eq. 13 are given as functions of the ratio $R(= C_I^0/C_{KI})$ as abscissas and the corresponding experimental values of E/n^2 from Table I are plotted as small circles. Here again the indication is clearly in close quantitative agreement with $j = 2$, or the complex ion I_3^-.

The existence of a complex of that composition has, from transference and distribution measurements, been known for a long time.[14] The results of this paper may, therefore, be regarded as a confirmation, by a quite new method, of the earlier conclusions.

A number of workers have determined the mass law constant for the equilibrium

$$I^- + I_2 = I_3^-$$
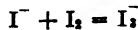
(13) This law is only approximate at 0.2 N, but it is a sufficiently good assumption for computing the relatively small term jtC of eq. 9.

(14) Bibliographies on the early work in this field are given by Bray and MacKay, THIS JOURNAL, **32**, 914 (1910), and by Jones and Kaplan, *ibid.*, **50**, 1845 (1928).

the most recent value being 1.40×10^{-3} at 25° for a total iodide concentration of 0.2 N as found by Jones and Kaplan.[14] A small proportion of the iodine in solution, therefore, exists in the form I_2 and the ratio R should, strictly, be corrected for this effect. The effect of the correction is shown in Fig. 5 by the dotted line diverging from the curve for $j = 2$, and is within the experimental error of the present series of measurements.

SUMMARY

The potential, E, of a galvanic cell consisting of two iodide-iodine electrodes at two different radii in a rotor has been measured at different speeds of rotation, n. Reproducible potentials were obtained only when radial temperature gradients were eliminated and the solutions in the cell were free from suspended particles. A series of measurements were made on solutions which had a constant concentration of potassium iodide, but varying concentrations of iodine. For each solution values of E/n^2 were found to be accurately constant through a range of values of n from 1800 to 7200 r. p. m. The ratio E/n^2 increases with the proportion of free iodine, a fact that is quantitatively accounted for by the presence in solution of the ionic complex I_3^-. The measurements also yielded a value of the transference numbers of potassium iodide in agreement with those obtained by the moving boundary method.

A DEVICE FOR THE MEASUREMENT OF ROTOR TEMPERATURE IN THE AIR-DRIVEN ULTRACENTRIFUGE

By PAUL GERARD ECKER,* JOSEF BLUM, and C. W. HIATT‡

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

An important factor in determining the accuracy of sedimentation constants obtained in the ultracentrifuge is the precision with which the temperature of the solution is known. An error of 0.4°C, for example, in estimating the temperature of the solution may result in an error of one percent in the measured sedimentation constant. In sedimentation equilibrium experiments, which require continuous operation of the centrifuge for several days, unsuspected variations of temperature may constitute an even more significant source of error.

In an effort to improve the accuracy of the air-driven ultracentrifuge, the authors have constructed a device which permits the measurement of rotor temperature while the centrifuge is operating at speeds as high as 60,000 r.p.m. The determinations are made with an apparent precision of ±0.02°C and are believed to represent the actual temperature of the solution within ±0.1°C or less.

The apparatus employed is shown in schematic form in Fig. 1. A Western Electric Type V 578 Thermistor, *Th*, is used as the temperature-sensitive element of the circuit.[1] The Thermistor employed is a small bead of semiconducting material having a resistance of about 75,000 ohms at room temperature and a temperature coefficient of about −5000 ohm per degree. One of the platinum leads from the Thermistor is attached to the body of the rotor, the other is led through an insulating tube to the bottom contact *J*2, where the circuit is completed through a flexible needle guide tip as described below.

Thermistor resistance measurements are made with the Wheatstone bridge shown in the diagram. The bridge current is supplied by a 1.5-v air-cell, *B*, and is reduced to a value of five microamp. by the rheostat *R*4. When the bridge is in balance, the current passing through the Thermistor is only 2.5 microamp.

Electrical contact between the bridge and the turbine shaft is made at *J*1 through the needle contact shown in Fig. 2. A steel sewing needle, *G*, is mounted on the top end of the shaft by the brass cap, *H*, and rotates with the shaft. The

tip of the needle presses against the concave bottom surface of an Oilite cylindrical brush, F, which is subjected to a downward pressure by the compression spring, E. The entire brush-mounting tube, B, may be adjusted in vertical
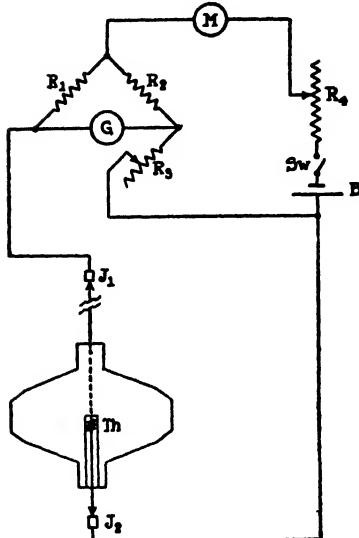
FIG. 1.   FIG. 2.

FIG. 1. Schematic diagram of the bridge circuit.

R1, R2—100,000-ohm wire wound precision resistors,
  R3—Shallcross No. 827 resistance box, 0 to 1,111,000 ohms in 100-ohm steps,
  R4—Wire wound rheostat, 200,000 ohms, with SPST switch (Sw) attached,
  Th—Western Electric Type V 578 thermistor,
  G—Leeds and Northrup mirror-type galvanometer,
  M—Weston microammeter, 0 to 20 microamps.,
  B—1.5-v. Eveready air-cell, Type A-1300,
  J1—Top contact (see Fig. 2),
  J2—Bottom contact (see Figs. 3 and 4).
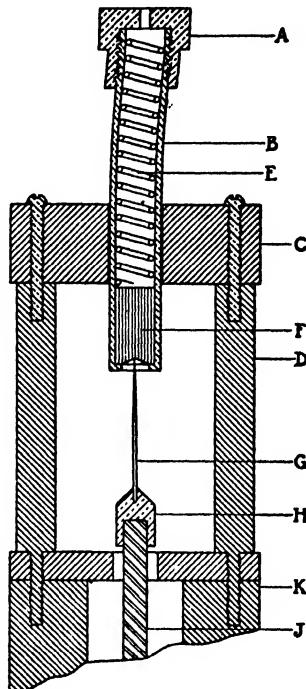
FIG. 2. The top contact.

position by sliding it up or down in the mounting frame, C, D. When placed so as to give proper pressure upon the needle tip, the tube is held by a set screw in the frame member, C. A hole in the brass cap, A, allows the introduction of small amounts of light oil occasionally to keep the Oilite brush lubricated. The arrangement shown affords a dependable, low resistance contact which func-

tions satisfactorily at all speeds of shaft rotation, shows very slight evidence of wear, and imparts only a minimal friction drag upon the shaft.

From this contact the current passes down the turbine shaft and through the body of the rotor to one side of the Thermistor, as shown in *A*, Fig. 3. The Thermistor is mounted in a soft copper sleeve, *B*, which is affixed to the end of an insulating Bakelite rod, *C*. One lead wire from the Thermistor bead is crimped into a slot in the copper sleeve, which is pressed tightly against the ma-
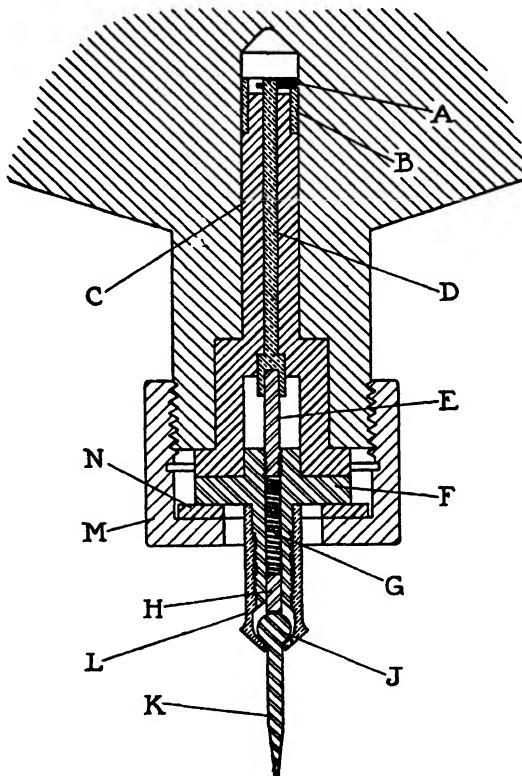
FIG. 3. The bottom contact and guide tip.

terial of the rotor. The lead wire from the other end of the Thermistor is pressed tightly into a slot on the end of the copper conducting rod, *D*, which is attached to the steel guide-tip assembly, *F, L*. The guide-tip assembly terminates in a steel needle tip, *K*, which is mounted in a ball joint, *J*. The compression spring, *G*, and plunger, *H*, maintain a constant pressure upon the spherical end of the needle and determine flexibility of the ball joint. The entire assembly is held firmly in place on the bottom of the rotor by the retaining cap, *M*, and fiber insulating washer, *N*.

When the rotor is lowered into operating position in the vacuum chamber,

the guide-tip assembly descends into the bearing receptacle depicted in Fig. 4. The needle passes with ample clearance through the cap, B, and brass safety bearing, C, and rests upon the top, concave surface of the spring-loaded Oilite brush, F. The brush itself, along with its compression spring, K, is mounted in the floating bearing structure, D, E, which is free to move laterally to center itself beneath the descending needle. Electrical contact is made through the brush, F, and the current is conducted through the bearing mount to the brass washer, G, the binding post, H, and thence returns to the bridge circuit. The entire assembly is held in a Bakelite receptacle, A, which is mounted rigidly on the bottom plate of the vacuum chamber. The safety bearing, C, does not enter
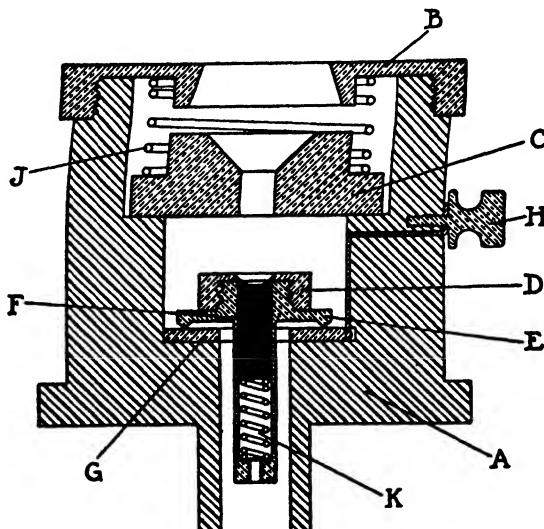
FIG. 4. The bottom bearing receptacle.

into the normal operation of the centrifuge, but is provided only to receive the weight of the rotor in the event of shaft breakage.

In addition to serving as an electrical contact, the needle functions as a guide tip to keep the rotor from precessing on its flexible shaft. By virtue of the ball-joint mounting, the needle is able to incline as required to keep its tip at all times on the center of rotation of the rotor. In so doing it acts in the same manner as the tool (called a "wiggler") used by machinists for precise centering operations. When the needle has sought and found the proper position for its tip upon the brush, it serves to guide the rotation of the rotor, there being sufficient rigidity at the ball joint to keep the rotor from precessing. Any slight change in the center of gravity of the rotor during rotation is rapidly accommodated by the needle, which merely inclines as required to bring its tip to the new center line of rotation. For optimal operation of the device it is necessary

that the compression springs (G, Fig. 3, and K, Fig. 4) be properly matched in strength, and it is desirable that the freedom of motion at the ball joint be restricted to the minimum required (about two degrees of arc in the present case).

The Thermistor was calibrated by immersing the entire mounting assembly in an oil bath at accurately known temperatures between 0° and 30°C. The calibration curve obtained was reproducible within 0.02°C. No tendency for the resistance value to drift with time was observed. The current used in measurement has been limited to 2.5 microamp., this being the minimal value for adequate galvanometer response with the present equipment. Electrical heating under these conditions appears to be entirely negligible. Tests of the total contact resistance in the circuit showed that this quantity was ten ohms or less at all speeds of rotation up to 60,000 r.p.m., with no tendency to increase with moderate wear. The low value of the contact resistance allows it to be ignored in the measurement of Thermistor resistance, which is 75,000 ohms or greater.

Resistance readings are taken continuously, or at arbitrary intervals during the operation of the centrifuge. When measured to the nearest 100 ohms, each resistance reading represents a temperature measurement which is reproducible within ±0.02°C. In conversion of resistance readings to temperature, which is done with reference to the calibration curve, the temperature values are rounded off to the nearest 0.1°C. After a more thorough investigation of the influence of gradients of temperature in the rotor, it may be possible to utilize the full precision of the apparatus and express temperature readings to the nearest 0.02°C. Efforts are being made at present to employ the Thermistor simultaneously as a thermoregulator to maintain the vacuum chamber and the rotor at a substantially constant temperature for sustained periods of operation.

PHOSPHORUS CONTENT OF OVALBUMIN AND OF SOME PRODUCTS OF ITS ENZYMATIC DEGRADATION

By GERTRUDE E. PERLMANN

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

As is well known[1,2], the phosphorus content of ovalbumin does not, in general correspond to an integral number of phosphorus atoms per mole of this protein if its molecular weight is taken as 44,000. About two years ago, Linderstrøm-Lang suggested to me that this lack of stoichiometry might be correlated with the occurrence, in ovalbumin, of two electrophoretically distinguishable components, A_1 and A_2[3], the relative abundance of which varies somewhat from one preparation to another but averages about 85 per cent A_1 and 15 per cent A_2. In the course of the electrophoretic studies carried out in this laboratory of the ovalbumin → plakalbumin transformation recently discovered by Linderstrøm-Lang and Ottesen[4-6], a modification of ovalbumin was encountered which crystallized in plates but differed electrophoretically from plakalbumin[7]. The solution from which these plate crystals were obtained was found to be contaminated with Gram-positive, rod-shaped bacteria, probably *B. subtilis*. In subsequent experiments a subculture from a single colony of this bacterial contaminant was kept in blood broth as the stock culture and was used as a source of enzymes for modifying ovalbumin. Phosphorus analyses of the proteins at different stages of enzymatic degradation were made. A comparison of these results with the electrophoretic analyses has made possible a test of Linderstrøm-Lang's suggestion.

The experimental procedure can be described in conjunction with the results summarized in the accompanying table. A sample of freshly prepared salt-free ovalbumin, having the composition given in line 1, cols. 3 and 4, and a phosphorus content shown in col. 9, was inoculated with a loop-full of freshly prepared broth culture. Aliquots were removed, at the times given in col. 1, for analyses, the results of which are reported in the corresponding line of the table. Most of the protein in each aliquot could be crystallized, and the crystal form is stated in the second column. On incubation of the solution at 37°C., a definite lag period was observed during which no changes of the ovalbumin occurred. The protein in the aliquot taken after 14 days incubation, however, crystallized in plates and had the same phosphorus content as the starting material. Since the non-protein nitrogen (col. 8) corresponded closely to that observed by the Danish workers for the ovalbumin → plakalbumin transformation[4-6], it may be concluded that at this stage the protein is present as essentially 'pure' plakalbumin, P. As previously noted[6], the reactions $A_1 \to P_1$ and $A_2 \to P_2$ appear

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to proceed at about the same rate and are accompanied by mobility changes that can be correlated with the loss of a glutamic and possibly aspartic acid residue in the $A \rightarrow P$ process. The components of the mixtures A_1, A_2, P_1, P_2 and Q are identified in the table on the basis of their mobilities, the values of which in a 0.1 ionic strength sodium phosphate buffer at pH 6.8 are given in parentheses. The validity of this identification has been checked in several instances by electrophoretic resolution of appropriate synthetic mixtures of the components.

On continued exposure to the bacteria, a component, Q, of low mobility appears in the patterns, and the percentage of non-protein nitrogen progressively increases while the protein phosphorus decreases. The lost phosphorus

Incubation at 37° C. (days)	Crystal form	Concentrations of electrophoretic components (per cent)					% Non-protein nitrogen	Mgm. phosphorus per gm. nitrogen	Atoms phosphorus per mole protein	
		A_1 (−5.9)	A_2 (−4.8)	P_1 (−5.5)	P_2 (−4.4)	Q (−3.6)			Observed*	Calculated from electrophoretic data
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
0	Needles	83.4	16.6					8.2_9	1.8_8	1.8_3
14	Plates			84.1	15.9		1.2	8.2_0	1.8_4	1.8_4
17	Plates			43.4	41.5	15.1	15.7	5.4_0	1.2_1	1.2_8
20	Plates			29.7	52.3	18.0	21.0	5.1_8	1.1_4	1.1_2
26	Plates			16.1	57.2	26.7	37.7	4.1_0	0.9_1	0.8_9
36	Plates			10.8	56.6	32.6	51.0	3.9_0	0.8_7	0.7_8

* Evaluated from the data of col. 9, assuming a protein molecular weight and nitrogen content of 44,000 and 15.76, respectively.

is recovered quantitatively in the non-protein fraction as inorganic phosphate. It is suggested that these effects may be due to the presence of other enzymes, which produce changes of the protein different from that responsible for the $A \rightarrow P$ process. It is clear that at least one phosphatase is included among these enzymes. Throughout this degradation, however, the residual protein remains crystallizable as plates. Moreover, the Q component is reasonably homogeneous electrophoretically. These observations suggest that Q is simply dephosphorylated plakalbumin, and that this dephosphorylation occurs before essentially complete degradation of plakalbumin by the non-specific proteolytic enzymes takes place. It is also clear now that the modification of ovalbumin originally observed in the contaminated solution[7] had proceeded somewhat beyond the plakalbumin stage.

If now the assumption is made that both A_1 and P_1 contain two atoms of phosphorus per mole of protein, A_2 and P_2 one atom per mole and that Q con-

tains no phosphorus, the phosphorus content of each preparation listed in the table can be computed with the aid of the electrophoretic analyses. The values thus computed (col. 11) are in excellent agreement with the actual determinations (col. 10), and afford a confirmation of Linderstrøm-Lang and Ottesen's hypothesis regarding the phosphorus content of ovalbumin[6]. Moreover, if the phosphorus is present in the protein as a negatively charged phosphate group, the loss of this charge on dephosphorylation should decrease the anodic mobility and, as can be seen from the table, this is observed experimentally. It is thus possible that the main difference between A_1 and A_2 and between P_1 and P_2 is in the group containing the phosphorus.

Work on a more direct test, namely, the phosphorus content of purified A_1 and A_2, is in progress; but the fractionation is proving to be a difficult one. The following observations have been of value throughout this work: preparations of ovalbumin have been stored as a paste under saturated ammonium sulphate at 3° for as long as eight years without a change in the electrophoretic pattern or of the phosphorus content. If a salt-free, isoelectric ovalbumin solution is sterilized by filtration through a 'COORS' bacteriological filter, it also can be stored unaltered with respect to its electrophoretic composition and its protein phosphorus. However, in the case of salt-free solutions, toluene is inadequate as a preservative. Solutions stored in this manner frequently become infected, and enzymatic changes occur which are similar to those here reported. It is quite possible that the changes observed by MacPherson, Moore and Longsworth[9] were of this type, and that the $A_1 \rightarrow A_2$ transformation described by them actually involved proteolysis and dephosphorylation as in the present case.

I wish to express my sincere thanks to Prof. K. Linderstrøm-Lang for valuable criticism, to Dr. L. G. Longsworth for help in preparing this manuscipt and to Dr. R. C. Lancefield for her advice throughout this investigation.

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FREE RADICALS DERIVED FROM TOCOPHEROL AND RELATED SUBSTANCES

By L. MICHAELIS and S. H. WOLLMAN*

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Tocopherol is known to exhibit two properties: It serves as a vitamin, and also as an antioxidant with respect to the autoxidation of unsaturated fatty acids. The latter property is shared with many substances of phenolic character. Although the mechanism of the antioxidant effect is not fully understood, and the mechanism of its effect as vitamin E is not understood at all, the suggestion as to some relationship of those two effects is almost inescapable. The vitamin effect may be closely related to the antioxidant effect, except of course for the fact that the more specific effect of the vitamin requires a special structure in addition to the general feature of being a substituted hydroquinone. It may be left undecided whether the specific structure is just to make it more fat-soluble or to adapt it to any function as a coenzyme to some enzyme.

Hydroquinone is an efficient antioxidant[1]. Although the mechanism of its action is not known in every respect, it can scarcely be doubted that this effect is in some way connected with its reversible oxidizability. However, also phenols with only one (or at least one unsubstituted) hydroxyl group are antioxidants[2]. Here no reversible oxidation comparable with that of hydroquinone can take place. The reversible oxidation of hydroquinone leads to quinone, by a bivalent oxidation passing throught the intermediate stage of a semiquinone. For monophenols, no such bivalent reversible oxidation is imaginable. However, a reversible univalent oxidation to a free radical is imaginable both for hydroquinone and for monophenols‡, including tocopherol. Such a radical would be a rather unstable compound. Ordinary oxidizing agents may not be able to produce the semiquinone radicals to any readily recognizable extent; yet, if a free radical may be produced only to a slight extent, not recognizable directly, the high energy content of the radical would make it a powerful reactant; just as the free OH radical, although never existing to any directly recognizable extent in an aqueous solution, has been recognized as a powerful reagent in many chain reactions.

However, any speculation about such free radicals is all too vague unless there is more direct evidence for their existence. It is the purpose of this paper

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* Special Research Fellow of the National Cancer Institute.

‡ At the present time, it will not be discussed whether even one unsubstituted hydroxyl group is necessary at all for the establishment of a free radical of comparable structure.

to produce such evidence. It is based on a method devised by G. N. Lewis[3, 4] and consists of the following procedure. The substance to be oxidized is dissolved in an organic solvent such as, at the temperature of liquid air, will, freeze to a homogeneous glass without crystallizing and is irradiated with ultraviolet light through quartz windows in a Dewar vessel. Such an irradiation may have two effects: one is, to raise the energy of some electron to a higher level. The spontaneous return of this electron to its ground level will be manifested by some luminescence, either fluorescence or phosphorescence of longer duration, according to conditions discussed by Lewis. In the second place, if there be an electron of sufficiently low ionization potential, the electron may knocked out altogether, a process comparable to oxidation by a chemical oxidizing agent. At the temperature of liquid air and in the rigid medium molecular collisions are inhibited. Free radicals, once created, will accumulate to a concentration far above that permissible by thermodynamics, provided
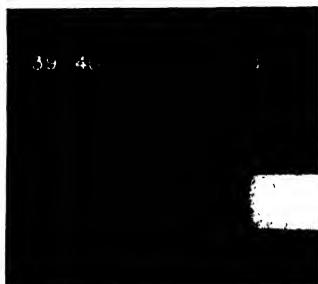
FIG. 1 shows the absorption spectrum of irradiated α-tocopherol at liquid air temperature, photographed with a spectrograph.

the electrons ejected are trapped in the molecules of the solvent and do not re-combine with the free radicals. In this case, no equilbrium in which the radical may be involved, can be established. Reactions siuch as dismutation, or dimerization of the radicals cannot occur. If the radical happens to be stable in so far as not to suffer a decay by a spontaneous unimolecular reaction (such as occurs in a radioactive atom), it will accumulate to a thermodynamically impermissible concentration. If the radical should be coloured, it could be seen in the frozen medium and remain as long as the temperature is kept low. On slightly warming up the solution the colour should disappear. This may be taken as evidence for the fact that the colour belongs to a compound capable of existence to a noticeable extent only under conditions where the establishment of chemical equilibria is inhibited*.

* According to Lewis and his associates, there may be still another effect: dissociation of a large molecule (such as tetraphenylhydrazin) either into two free radicals, or into a positive and a negative ion. Considering the structure of the compounds

The colour produced in this way can, in suitable cases, be compared with the colour of free radicals produced by chemical oxidation. In fact, the absorption spectrum of the compound generated by either method was found to be identical[3] on working with such substances as asymmetrical dimethyl-p-phenylene diamine, or tritolylamine[5, 6].

In this paper we shall describe the absorption spectra of several coloured substances considered as free semiquinone radicals prepared in this way from substances related to tocopherol. They are all produced by irradiation of a solution in a mixture of ether, ethanol, and pentane†, in the volume proportions 5:2:5, respectively, with an ultraviolet lamp for the duration of a few minutes
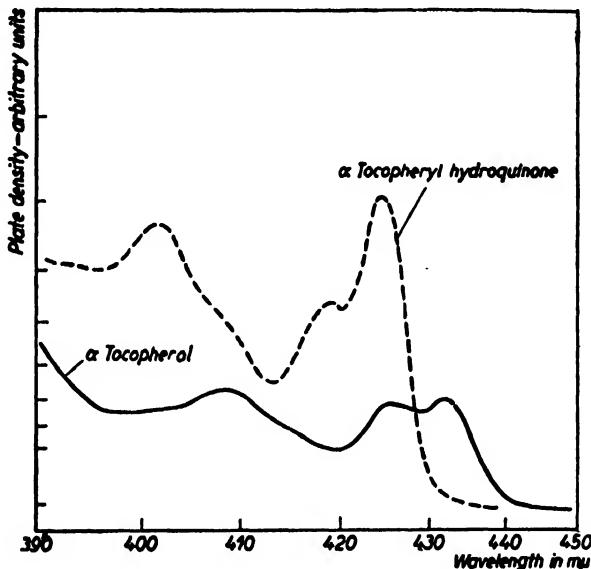
Fig. 2 shows tracings, obtained with a recording microphotometer, of the spectrum of irradiated α-tocopherol, and of irradiated α-tocopherylhydroquinone‡.

to about twenty minutes. Although the method is not suitable in its present form to tell anything about the yield, it may be stated, that the radical of tocopherol is produced with ease to a readily recognizable extent.

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investigated, the possibility of such effects may be disregarded here. The fact that all the spectra obtained from the various compounds are similar, is further evidence as to the absence of any essential photodecomposition.

† G. N. Lewis recommends isopentane. We had no trouble with ordinary commercial pentane. If the mixed solvent shows any inclination to crystallize at liquid air temperature, it can be corrected by adding slightly more ether.

‡ The authors are indebted to the Sun Chemical Company, New York, for their permission to use their recording microphotometer.

Among the substances irradiated during this experimental study there is, first of all, hydroquinone. It is irradiated, then the decay of the phosphorescence is awaited (usually several seconds), without lifting the vessel out of the liquid air environment. Now the colour in transmitted light is observed. It is yellow, its absorption spectrum consists of several bands in the visible, the maxima of which are reproduced in Fig. 3. The yellow substance is not quinone. Firstly, its absorption spectrum is different from that of quinone; secondly, this colour vanishes on slightly warming up the frozen mixture. In addition, a spectrum of the same character is produced in this way from hydroquinone-monomethyl ether. This, of course, cannot be oxidized to the level of a regular quinone but there is no reason why it should not be oxidized to the level of a semiquinone.

Of the various tocopherols, samples of pure α, δ, and γ tocopherol* and several samples of commercially available α-tocopherol were compared. The latter showed the same behaviour as the pure α-tocopherol, whereas the δ and γ
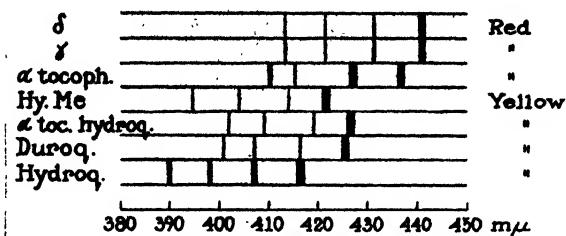
FIG. 3. shows the location of the absorption bands after irradiation as obtained according both to photographs such as Fig. 1 and to tracings such as Fig. 2.

compound showed, after irradiation, absorption bands slightly different from the α-compound. Whereas the colour of the radicals from hydroquinone and its methyl-ether are yellow, that of all the tocopherols is red, of slightly orange tint. This difference corresponds to the location of the absorption bands in Fig. 3.

The problem arises whether this "oxidation" by irradiation is a reversible one. Only in this case the substance could serve in metabolism as something analogous to a coenzyme of an oxidative enzyme. When tocopherol is chemically oxidized (say by ferric chloride), the first oxidation product obtainable is a quinone, tocopherylquinone[7], which cannot be re-reduced directly to the original tocopherol because the phytol side-ring is opened to make the quinone. When this quinone is reduced to its corresponding hydroquinone, and this "tocopherylhydroquinone" is irradiated under proper conditions, the absorp-

*We owe these to the courtesy of Distillation Products Corporation, Rochester, N. Y.
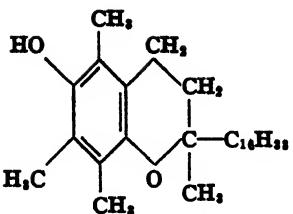
α-tocopherol, parent substance
of radical No. 3 in Fig. 3
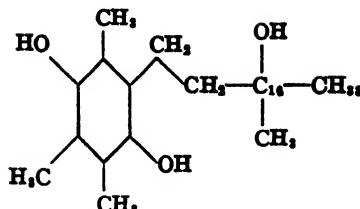
α-tocopherylhydroquinone, parent
substance of radical No. 4 in Fig. 3

tion spectrum of the free radical is different from that produced by the irradiated tocopherol itself. It resembles, with its yellow colour, more that of the hydroquinone-methyl-ether. Hereby it is shown that the red radical produced from tocopherol does not involve the opening of the phytol side-ring. The preservation of the free radical will also be aided by the fact that the opening of the phytol ring represents a hydrolysis which cannot occur in the absence of water. There is, then, no reason, why the univalent oxidation of tocopherol, especially in a non-aqueous solvent, should not be reversible.

SUMMARY

Tocopherol, dissolved in a suitable mixture of organic solvents such as will, at the temperature of liquid air, form a homogeneous glass, is irradiated with ultraviolet light. A red colour is developed which disappears at slightly higher temperature. Similar observations are made with some other substances related to hydroquinones. The coloured substance is interpreted as a free semiquinone radical. Its possible function for the vitamine and the antioxidant effect of tocopherol is discussed.

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EXPERIMENTAL DISSEMINATED ENCEPHALOMYELITIS IN WHITE MICE

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PLATES 19 AND 20

Disseminated demyelinating encephalomyelitis occurs not infrequently as a sequel to, or during convalescence from, clinically apparent infection with a number of different viruses, and rarely after vaccination against smallpox, rabies, and other viral diseases. A similar pathological picture characterizes several neurological maladies, for example, multiple and diffuse sclerosis, Schilder's disease, leucoencephalitis, neuromyelitis optica, and a score of other encephalitides, each regarded by the one who first named it as a nosological entity. Several investigators, however, hold that several or all the latter demyelinating affections belong in the same groups of essentially similar histo-pathological processes (Ferraro (1); Putnam (2); Roizin, Helfand, and Moore (3); and others).

Although the problem of the etiology of the demyelinating diseases has been studied during the past century and considerable experimental research has been carried on, there is still no solution. A new impetus, nevertheless, has been the successful experimental production in laboratory animals of neurological syndromes accompanied by histopathological changes in the central nervous system similar to those found in the acute demyelinating affections.

The earliest reports of successes were those in 1933–35 of Rivers, Sprunt, and Berry (4) and Rivers and Schwentker (5) who injected monkeys repeatedly over a period of months with brain tissue obtained from apparently normal rabbits. The first paper recorded that of eight animals receiving 14 to 93 intramuscular injections thrice weekly, two reacted with demyelinating encephalomyelitis, one after the 52nd and the other after the 84th inoculation. The second report showed that of eight monkeys similarly treated 46 to 85 times, seven gave positive results. This finding was promptly confirmed by Ferraro and Jervis (6) who introduced rabbit brain into monkeys 29 to 103 times over a period of 112 to 405 days; the animals first showed neurological signs after 3 to 13 months. Ferraro (1) in 1944, concluded that the reaction in the nervous system is of an allergic nature—a view which found active support so that commonly the experimental disease, and occasionally the human acute demyelinating

affections are now called "allergic (or isoallergic) encephalomyelitis."[1] In other words, the brain tissue used for producing the encephalomyelitis is considered to be a sensitizing antigen.

Another advance was gained by the addition to brain tissue of what is now known as the "Freund adjuvant" for the purpose of bringing about experimental disease more rapidly and regularly. Freund and McDermott (8) demonstrated that a mixture of lanolin-like substances, paraffin oil, and killed tubercle bacilli, induced a prompt and high antibody response when added to a wide variety of antigens, and Kopeloff and Kopeloff (9) showed later that by use of the adjuvant antibrain antibody could also be elicited, for example, in the blood of monkeys receiving injections of sheep brain. Morgan (10) and Kabat, Wolf, and Bezer (11) at the same time and independently, adding the adjuvant to homologous and heterologous cerebral tissue, induced encephalomyelitis in monkeys by only a few injections, even a single one, and neurological signs appeared in days instead of months. Later Ferraro and Cazzullo (12) demonstrated that chronic types of encephalomyelitis could be brought on in monkeys given reduced amounts of the antigen. Such types displayed histopathological changes similar in many respects to the lesions noted in acute human demyelinating diseases (12).

A technical advance was the finding that rabbits (13, 14) and guinea pigs (14–16) could be utilized instead of monkeys. The experimental production of neurological signs and lesions in any species, whether monkey, rabbit, or guinea pig, was found, however, to be unpredictable. Now and again half the injected number, sometimes less than half, reacted positively. Furthermore, in those animals which gave positive results the number and spacing of injections, and the length of time after exposure before the onset of the syndrome varied within extraordinarily wide limits.

Disseminated encephalomyelitis of the type now under discussion has not as yet been induced in albino mice, despite several attempts (11, 14, 15). If mice could be proved susceptible, especially, if they reacted with some regularity and to nearly the same degree, an advantage would be gained for the study of the experimental disease. The results of the present investigation[2] show that acute, subacute, and possibly, chronic disseminated encephalomyelitis can be readily produced in mice merely by intramuscular and subcutaneous injection of homologous brain tissue mixed with an adjuvant modified in a way to be described. Moreover at least one of the causes for the failures of others to produce encephalomyelitis in white mice was revealed in the fact that different strains of mice differ markedly in susceptibility.

Methods and Materials

Mice.—Albino mice of the Swiss and the Rockefeller Institute strains, aged 6 or 7 weeks were selected from apparently healthy stocks. Mice of the Swiss strain proved to be much more responsive to injections of brain tissue and hence the experiments were mainly carried out with this strain.

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[1] For a discussion and review of literature on allergy in the nervous system reference should be made to Ferraro (1), Stevenson and Alvord (7), and Hurst (22).

[2] A preliminary report of these investigations has already been published (17).

Adjuvant.—As originally devised by Freund (8) the adjuvant consisted of killed tubercle bacilli and paraffin oil in a water-oil emulsion. To secure proper emulsification, various agents were used, chief among them "falba" and "aquaphor," both of them proprietary materials, the former an adsorption base derived from lanolin, the latter an ointment base, from a mixture of wool fat and hydrocarbons. They have been employed by earlier investigators for the purpose of enhancing the experimental production of encephalomyelitis.

In the present studies, the preparation of the adjuvant was modified. No special emulsifier was added to the autoclaved tubercle bacilli, and for light paraffin oil, that of heavy type (liquid petrolatum, soconal)[3] was substituted. This was used not only as an emulsifier but also for its possible enhancing action on the antigenicity (8, 15) of the mouse brain tissue. The tubercle bacilli were of human type, virulent strain, H37Rv (18), stored acetone-dried, and autoclaved for 15 minutes at 15 pounds pressure before use.[4] The mixture of materials was homogenized in a small sized Waring blendor, the end product being a thick emulsion which could be stored for several weeks at 4–5°C. without evident deterioration.

The proportions of the materials in the mixtures were changed as time went on and the antigen finally was made up as follows: Mouse brains derived from normal appearing Rockefeller Institute or Swiss strain mice, 10 gm.; autoclaved tubercle bacilli, 20 mg.; liquid petrolatum, heavy type (soconal), 50 ml.; and 0.85 per cent saline solution, 50 ml.

Dosage.—Of the mouse brain–adjuvant mixture, 0.3 ml. was injected intramuscularly into the thigh. When more than two or three intramuscular injections were given, the later ones were introduced subcutaneously because the nodules which developed at the site of inoculation prevented a reinoculation of the same area. The way in which the experiments were conducted made it possible to select for the larger number of exposures to the antigen the more resistant animals of a given group. For example, the reactors to one injection did not receive any further inoculations; the non-reactors were given a second dose, and so on, those animals which showed no effects being inoculated up to 5 or 6 times, that is to say until they responded or consistently resisted, as happened in a few instances.

Histopathological Studies.—60 to 100 sections obtained from various parts of the brain and cord of each injected mouse were studied for changes. Some of each series of sections from the same fragment of tissue were stained by the erythrosin-Azur I, hematoxylin-eosin, and Loyez' myelin methods respectively; also by the Bodian silver impregnation method and sometimes by the Ranson.

EXPERIMENTAL

The syndrome of disseminated encephalomyelitis accompanied by characteristic histopathological changes, which will be described in the next sections of this paper, was induced in Swiss strain mice by means of peripheral (intramuscular and subcutaneous) injections of normal mouse brain mixed with adjuvant, as just described.

Symptom Complex

Local Reaction.—In every instance in which the adjuvant, heavy liquid petrolatum by itself or together with killed tubercle bacilli, was injected, a nodule promptly formed at the site of inoculation. The nodule was absorbed

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[3] Dr. J. Freund (21) in a repetition of some of the tests here described succeeded in producing the characteristic encephalomyelitis by substituting light for heavy liquid petrolatum.

[4] The writers are indebted to Dr. G. Middlebrook, Dr. C. Pierce, and Dr. M. W. Chase for these materials.

with difficulty and hence persisted for several weeks. It contained grumous and fatty material which was probably unabsorbed inoculum. Generally speaking, these nodular masses can be looked upon as sterile or "cold" abscesses. Since they were of considerable size, and were situated in the thigh muscles, they often affected the movements of the animal—a fact which should be reckoned with in estimating the neurological significance of a peculiar gait.

Constitutional Reaction.—Dyspnea, occasionally accompanied by wheezing respiration, was commonly a manifestation of the disorder. The type of breathing was similar to that in allergic bronchial asthma. The mice showed this difficulty intermittently, periods of a few hours to several days intervening before the attack was renewed. Sneezing and pawing of the nose also accompanied the stridor.

In an exceptional case, two mice receiving one injection of brain tissue–adjuvant mixture showed on the 2nd day dyspnea accompanied by wheezing respiration and no other sign except ruffled fur. This endured for 2 and 3 days, after which the animals recovered their former apparently normal, state. On the 8th and 9th day, respectively, both lapsed into the characteristic neurological state, soon to be described.

The constitutional disorder was notable for (a) wide divergence in time before the onset of illness; (b) varied symptomatology; and (c) repeated relapses arising in the course of an enduring yet ordinarily non-fatal affection. The earliest indications of disease were ruffled fur; considerable loss of weight; slowness of movements and weakness of the extremities. Within a few days intensification of the neurological symptoms was noted, accompanied by generalized coarse tremors; ataxia; excited movements in some mice and apathy in others. Paresis of the limbs occurred often but paralysis, usually spastic in type was infrequently seen, as was paralysis of the rectum and bladder. Certain animals exhibited alternating periods of excitement and somnolence or circling; others developed a long enduring catatonic-like posture in which the mouse stood partially erect, resting on the pads of the hind feet with forepaws flexed on the chest. Still others walked with arched or hunched backs on tiptoes, a mincing gait. Generalized convulsive movements were observed but rarely. A single mouse might exhibit all, or only one or more, of the described patterns of behavior during an attack. Clearly, the marked diversity of reaction depended for its expression on the area of the brain and cord which was damaged. During relapses the signs were not always identical or even resembled those seen in the attack immediately preceding. Thus the main sign of one attack might be wheezy breathing; of a relapse, paralyses of the limbs. The usual course of the experimental disease tended, therefore, toward a state of chronicity marked incidentally by relapses of variable duration and terminated usually by recovery, and infrequently by persistent paralysis of one or more limbs. Death supervened in about 5 per cent of inoculated animals and then, as a rule, during the early stages of the first attack. [5]

Histopathological Picture.—The earliest histopathological indications consisted of vascular changes; a relatively slight degree of intramural and perivascular infiltration extending out into the parenchyma. Such lesions were scattered here and there throughout the brain, especially the caudal part, and the cord. The infiltrating elements were chiefly mononuclear and polymorphonuclear leucocytes including some eosinophiles.

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[5] The possible production of encephalomyelitis by means of mixtures of killed tubercle bacilli, petrolatum, and various fractions of mouse brain tissue is under investigation; the lipid material was the first fraction to be tested.

The picture seen in the fully developed encephalomyelitis revealed a greater degree of vascular reaction in respect both to the number of vessels involved and to the cellular reaction in and around them (Figs. 1, 2, 6, 10–12). The infiltrating cells in and about the vessels and especially in the parenchyma were not only leucocytes and plasma cells but also microglia, including rod and compound granular (gitter) cells (Figs. 3 and 8). No giant cells could be detected. Associated with the vascular reaction, which apparently was the essential pathological process, were disseminated petechial hemorrhages (Fig. 4), and the formation of thrombi of hyaline and leucocytic types (Fig. 2). The changes were more prominent in the white matter than in the gray, and in the mesencephalon, cerebellum, and spinal cord more than elsewhere in the central nervous system. Degeneration of nerve cells (Figs. 3 and 7) and of Purkinje cells (Fig. 12) was found in scattered areas in these regions but neuronal necrosis was rare or absent. In addition, areas of agglomerations mostly of mononuclear, less of microglial cells, and still less of polymorphonuclear leucocytes and plasma cells (Fig. 9) were seen, as were disseminated areas of diffuse glial cell infiltrations (Fig. 8).

In animals in which the signs of active illness endured for 3 or 4 weeks demyelination was met with more often than in the acute cases of shorter duration. Myelinolytic areas were found more commonly in the parenchyma (Figs. 5 and 7) than perivascularly (Fig. 6) and involved the myelin sheaths of fibres with or without destruction of the axons themselves. One could also see in certain animals irregularly outlined coalescing areas of demyelination. The scattered glioses or glial scars so prominent in chronic multiple sclerosis were not in evidence in the present study.

In recovered mice killed for the purpose of histopathological examination—the possibility should be considered of an impending relapse had the animal been allowed to survive—the brain and cord still showed evidence of inflammatory reaction. Here and there a mild degree of the vascular reaction could be seen and in some areas a slight, diffuse glial infiltration.

The leptomeninges were not always involved; when positive they showed a characteristically spotty infiltration with mononuclear and polymorphonuclear leucocytes predominating. In such discrete areas, blood vessels were also damaged in the characteristic way as described. The choroid plexus rarely exhibited mild changes of similar types of infiltration. The subependymal areas were at times the sites of massive infiltrations (Fig. 2) which also involved the ependymal lining.

In the mice which reacted only with respiratory symptoms no changes were found in the central nervous system. The lungs observed at the height of a dyspneic attack were noted as pale, partially collapsed, and having an increased consistency—a picture which was similar to that seen in anaphylactic reactions of mice (19).

The persistent nodules which developed locally after intramuscular or subcutaneous injection were found to contain a mass of cells of the mononuclear series, polymorphonuclear leucocytes, epithelioid cells, and an occasional multinucleated giant cell, along with amorphous material and broken down cells. This entire mass was held together in a firm encapsulated structure honeycombed by strands of newly formed fibrous tissue.

To summarize: The histopathological picture in murine experimental disseminated encephalomyelitis was not essentially different from that seen earlier in monkeys (20), rabbits (13), and guinea pigs (15, 16). The local lesion at the site of inoculation was characteristic of a cold abscess, or a foreign body cutaneous reaction in which one found epithelioid and giant cells. The changes in the brain and cord related to a primary vascular reaction followed by productive inflammation involving mesodermal-glial elements and in later cases, a certain but not a marked degree of gliosis and of demyelination. It remains for further

study of chronic cases of the experimental disease—those of many months' duration and more sustained exposure to the antigen—to determine whether

TABLE I

An Experiment Illustrating the Response of Swiss Strain Mice to Injection of Normal Mouse Brain Plus Adjuvants

Mouse No.	No. and route of injection (0.3 ml. each)	Time of onset	Time after first injection			Signs		Lesions in CNS	Remarks
			Killed	Died	Survived	Neurological	Respiratory		
		days	*days*	*days*					
1	3 IM; 1 SC	17	46			P	P	+++	Three relapses
2	" "	33			Yes	P	A		Two "
3	" "	33			"	P	A		Two "
4	3 IM	16	17			P	P	++	Lesions shown in Fig. 10
5	" "	18	33			P	P	+++	Paralyzed 15 days
6	" "	18		43		P	P	++	Six relapses
7	" "	18		20		P	P	+++	Lesions shown in Fig. 4
8	" "		21			A	A	++	Observed for lesions before signs
9	" "		21			A	A	+++	" "
10	" "	21	42			P	P	+++	Ill 21 consecutive days
11	" "	21	54			P	A	+	Three relapses, then recovery 7 days; rare vascular lesion only
12	" "	21	54			P	P	++	Two relapses
13	3 IM; 1 SC	22			Yes	P	A		Three relapses; recovery 30+ days
14	" "	25	54			P	P	++	Ill 29 consecutive days
15	" "	27	54			P	P	None	Recovery 27 days
16	3 IM; 2 SC	42		79		P	P	n.h.	Ill 37 consecutive days
17	" "	42			Yes	P	A		Ill 33 consecutive days, recovery 7+ days
18	" "	54			"	P	A		Two relapses
19	" "	112		113		P	A		Ill only 1 day
20	" "				Yes				No signs (120+ days' observation)

IM, intramuscular; SC, subcutaneous; +, ++, +++, arbitrary units denoting degree of involvement of central nervous system (CNS); A, no physical signs detected; P, signs present; 30+ days, animal still ill or apparently well up to the time of writing; n.h., no histopathologic study made.

glioses, glial scarring, and demyelination take place, such as are the indicators of demyelinating diseases as they occur in nature (*cf.* Ferraro and Cazzuilo (12)).

Enumeration of Results.—The results set down in Table I were obtained in a single experiment in which normal mouse brain plus liquid petrolatum and tubercle bacilli as adjuvant comprised the antigen. The object of this tabulation is to give an informative picture of the response to the test. There

were additional experiments, not tabulated, which included 30 Swiss strain mice. The results of them all will be briefly set forth.

Encephalomyelitis was produced after one to six injections of the antigen. Of fifty mice, two gave positive results after only one inoculation of antigen and five after two. The largest number, namely eighteen, was positive after three injections; the next largest number, thirteen, after four. Only three mice responded to five doses and two to six. Three animals failed to show any visible effect even though five or six inoculations were given. Four animals were killed from 13 to 21 days after receiving two or three doses of antigen, before any clear indication of illness, and three of them exhibited histopathological lesions characteristic of disseminated encephalomyelitis.

The earliest period of time noted before onset of definite neurological signs was 9 days after the first treatment or 2 days after the second, and the longest, a rare instance, 112 days after the first, and 7 days after the sixth. The earliest development of respiratory signs of sneezing and wheezing respiration which later was followed by a definite neurological syndrome was first observed 2 days after a single injection of antigen. The majority of the mice recovered, only to relapse after a variable time. Quiescent periods lasted from 1 to 15 or 20 days. The number of such relapses over a period of 133 days was found to be six or less. The duration of illness, whether a primary attack or a relapse, varied from 1 to more than 30 days. Since most of the mice were killed during the active stage of the syndrome it is difficult to measure precisely the total number of days in the longest period of continuous illness.

To sum up the results on Swiss strain mice, it appears that disseminated encephalomyelitis could as a rule, be set up in them in a shorter time and with fewer exposures than in the other species of animals hitherto reported upon. Thus some mice exhibited signs of dyspnea and wheezy respiration within 2 days after a single inoculation; the majority showed a definite neurological syndrome within 9 to 21 days after two or three injections and only three of fifty animals failed to respond to the injections. A well defined individual resistance existed among the Swiss strain mice; now and again one reacted positively after one or two exposures to brain tissue–adjuvant mixture whereas others did so only after several injections given over a period of 2 or 3 months.

Host Specificity.—As already stated two inbred strains of mice, the Swiss and the Rockefeller Institute strains, were tested for their relative susceptibilities to development of encephalomyelitis following injections of mixtures of mouse brain and adjuvant.

Of fifteen Rockefeller Institute strain mice three received two intramuscular and one a subcutaneous injection of active material containing normal mouse brain mixed with adjuvant, and twelve were given four intramuscular and three subcutaneous inoculations. Of the fifteen animals only one, and that one of the first group, reacted with characteristic signs, on the 30th day after the first of three injections. This mouse was killed and typical lesions were found in its brain and cord (Fig. 7). In contrast, the same inoculum induced characteristic disseminated encephalomyelitis in nineteen of twenty Swiss strain mice after three intramuscular injections in nine mice and three intramuscular with additional one to two subcutaneous ones in the remaining ten; the malady appearing from 16 to 112 days after the first inoculation (Table I).

A wide difference was found, therefore, in the susceptibility of the two strains of mice; the Swiss strain were highly susceptible while the Rockefeller Institute

ones were relatively resistant. Not only was there this variation in strain but, as revealed in Table I, there were also differences among animals of the same Swiss strain some succumbing promptly upon a few inoculations whereas others did not respond at all or only after many weeks and after several exposures to the antigen.

Transmissibility of the Agent in Series.—The agent responsible for the experimental encephalomyelitis was found not to be serially transmissible in normal animals; it appeared not to be an infective, multiplying agent, as the results of the following tests showed.

The central nervous tissues of each of three mice killed at the height of the encephalomelitic reaction were suspended in a 10^{-1} dilution in physiological saline solution. 0.03 ml. of the preparations was injected intracerebrally into each of ten Swiss strain mice for each sample, or into thirty for all. None of the mice exhibited signs of illness.

Though no infective agent was demonstrated in Swiss strain mice an additional test was made with mice of the Rockefeller Institute strain, animals employed in this laboratory at at the time for experimental work on various encephalitis viruses. Each of fifteen normal animals was given 0.03 ml. intracerebrally of the active mouse brain–adjuvant mixture, and fif- others received 0.3 ml. peripherally (intramuscularly). No untoward effect was noted nor were any significant lesions produced except for the familiar nodules at the site of inoculation.

A third series of experiments involved the use of an acetone-ether "lipid" fraction obtained from normal mouse brain having a concentration of lipid of 20 mg./ml.[5] The aim here was an activation of a possible latent virus or other type of infective agent which might be present in the stock Swiss strain mice. Twenty animals were injected intramuscularly with 0.3 ml. of the lipid. The same animals received a second series of inoculations containing this material emulsified with equal parts of heavy liquid petrolatum. Each mouse was inoculated eight times but no observable signs or lesions could be discerned in the central nervous system during 139 or more days of observation. A second group of twenty mice received six intramuscular inoculations of the lipid fraction mixed with the adjuvant containing petrolatum and killed tubercle bacilli, and none of these showed signs of illness.

Repeated Exposure to the Active Agent.—A single injection of the mixture containing mouse brain–adjuvant failed, as a rule, to give a positive result; repeated exposure to this material was found to be essential.

Twenty Swiss strain mice received a single dose of 0.3 ml. of active antigen intramuscularly. Although the familiar nodules were formed at the site of inoculation, only one animal became ill, on the 54th day, showing signs of encephalomyelitis. On the other hand, positive results in nineteen of twenty mice, were obtained upon repeated injections of the same material (Table I).

Rôle of Killed Tubercle Bacilli as Adjuvant.—The enhancement of sensitization by an antigen through the adjuvant action of killed tubercle bacilli (Freund (8)) has been regarded hitherto as essential for producing disseminated encephalomyelitis readily. Killed tubercle bacilli were also found necessary to the affection produced in mice, as the following experiment makes clear.

Nineteen Swiss strain mice were given six intramuscular injections of an emulsion containing 25 ml. of heavy liquid petrolatum and 5 gm. normal mouse brain in 25 ml. of 0.85 per cent

saline soluuon; tubercle bacilli had not been added. No sign of illness was noticed; yet the emulsion used contained all the ingredients, except for the bacilli, which were present in mixtures producing positive results.

It is of interest that the liquid petrolatum which was added to normal brain tissue in the foregoing test did not suffice of itself to confer encephalitogenic power, even though it is known to promote antibody formation or antigenicity (Freund (8)). What may have been required was a higher degree of sensitization —if this indeed was the process involved—resulting from the presence in the inoculum of either killed tubercle bacilli as such or paraffin oil in addition to the bacilli.

DISCUSSION

Swiss strain mice, repeatedly injected intramuscularly and subcutaneously with a mixture of normal mouse brain and adjuvant developed symptoms and histopathological lesions of a disseminated encephalomyelitis like those of the encephalopathies induced by similar methods in monkeys, rabbits, and guinea pigs. Fewer injections were needed and of a simpler antigen, than in the case of the other animals mentioned; the experimental disease revealed itself in a shorter time; acute, subacute, or chronic forms could be readily produced; a larger proportion of inoculated mice became affected within a relatively brief time; and finally, the lesions induced were as marked, at least, as those found in the other species, from all of which it would appear that the mouse as experimental animal has certain advantages, apart from more convenient handling, lesser cost, and greater availability. It should be pointed out, however, that demyelination although met with occasionally in the mouse, now perivascularly, now parenchymally, and then in both sites, was not a prominent lesion. Demyelination has not always been seen in guinea pigs (15), although when it occurred in this species it was more often found, as in the mouse, in the later stages of the experimental affection (21). A study is planned of mice which have been kept under the influence of the active encephalitogenic mixture for many months, to determine whether more extensive demyelination and glial scarring develop.

The opinion has been expressed that the histopathological changes found in the various diseases included in the group of human demyelinating encephalopathies are essentially similar (1–3, 20, 22); and several investigators (1–7, 11–13, 20) have compared the lesions induced experimentally in various animal species with those of the human acute demyelinating diseases. There is good agreement on the resemblance of the pathological picture seen in man to that in the lower animals. No uniformity of opinion exists, however, as to whether the experimentally induced lesions resemble those of the human chronic encephalitides, multiple and diffuse sclerosis for example. In this relation, Wolf, Kabat, and Bezer (20) and Ferraro and Cazzullo (12) and others,

have stressed how individual is the reaction of an animal, the differences resulting from the duration of active illness and the number of relapses. The lesions in the mouse as disclosed by the present experiments resemble those in the species hitherto employed, and since the picture in the latter is similar to that observed in the natural disease, it would appear that study of the mouse can help in elucidation of some of the problems of the encephalopathies. The *demonstration of strain differences as well as of individual resistance to the development of disseminated encephalomyelitis has interest because of the known differences in the susceptibility of human beings to certain demyelinating diseases (Hurst (22)).*

The mouse may conceivably prove useful in the study of allergy in the nervous system. The present findings support the view that the experimental affection is an allergic, or isoallergic reaction to the injected material. The dyspneic, wheezy respiration and sneezing and pawing at the nose, occurring at the height of the reaction, are not unlike the signs of anaphylaxis in mice (19, 23). The fact that repeated injection with brain tissue–adjuvant mixture was necessary, a single inoculation being generally without effect, suggests sensitization (7). Furthermore, the development of encephalomyelitis was favored by the use of material which is known to enhance antibody formation. Whether the mixtures used excite an actual increase of antibrain antibody in the brain of mice is a problem awaiting further study as is that of a possible correlation between relapses and a recurring rise and fall of the level of this antibody. It may be that mice can be employed for investigations on the inhibition or destruction of the encephalitogenic factor present in normal brain.

CONCLUSIONS

Disseminated encephalomyelitis was readily induced in mice of the Swiss strain by means of repeated intramuscular and subcutaneous injections of apparently normal mouse brain mixed with an adjuvant. The latter consisted of autoclaved virulent tubercle bacilli and heavy liquid petrolatum, a modification of the Freund adjuvant.

The syndrome and the histopathological picture of the induced malady were essentially similar to those in monkeys, rabbits, and guinea pigs, previously reported by others. Certain exceptional characteristics of the affection, as occurring in mice, suggest that they may be the animals of choice for its study as well as for that of other encephalitides. Not only were the signs indicative of marked involvement of the central nervous system but also of the respiratory mechanism, and only a few injections of mouse brain–adjuvant mixture were required to evoke the neurological symptom complex in almost every animal.

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EXPLANATION OF PLATES

PLATE 19

FIG. 1. Mouse 2-5B, pons-medulla. Mural, perivascular, and extravascular infiltration. Hematoxylin-eosin stain. × 514. (Compare with Figs. 2, 3, 6, 10 and 11.)

FIG. 2. Mouse 7-5B, posterior colliculus. Extensive lesion chiefly perivascular, mural, and extravascular infiltration; diffuse glial infiltration of parenchyma and vascular thrombosis. Hematoxylin-eosin stain. × 135. (Compare with Figs. 10 and 11.)

FIG. 3. Mouse 2-5B, thalamus. Neuronal shrinkage and degeneration but no necrosis; also an area of vascular infiltration with polymorphonuclear and mononuclear leucocytes. Hematoxylin-eosin stain. × 308. (Compare with Fig. 7.)

FIG. 4. Mouse 7-5E, floor of fourth ventricle. Numerous petechial hemorrhages. Erythrosin-Azur I stain. × 128.

FIG. 5. Mouse 7-5B, white matter upper cervical cord. Destroyed and demyelinated axons. Bodian silver impregnation. × 502. (Compare with Figs. 6 and 7.)

FIG. 6. Mouse 9-5E, medulla. Perivascular area of demyelination. Bodian silver impregnation. × 128.
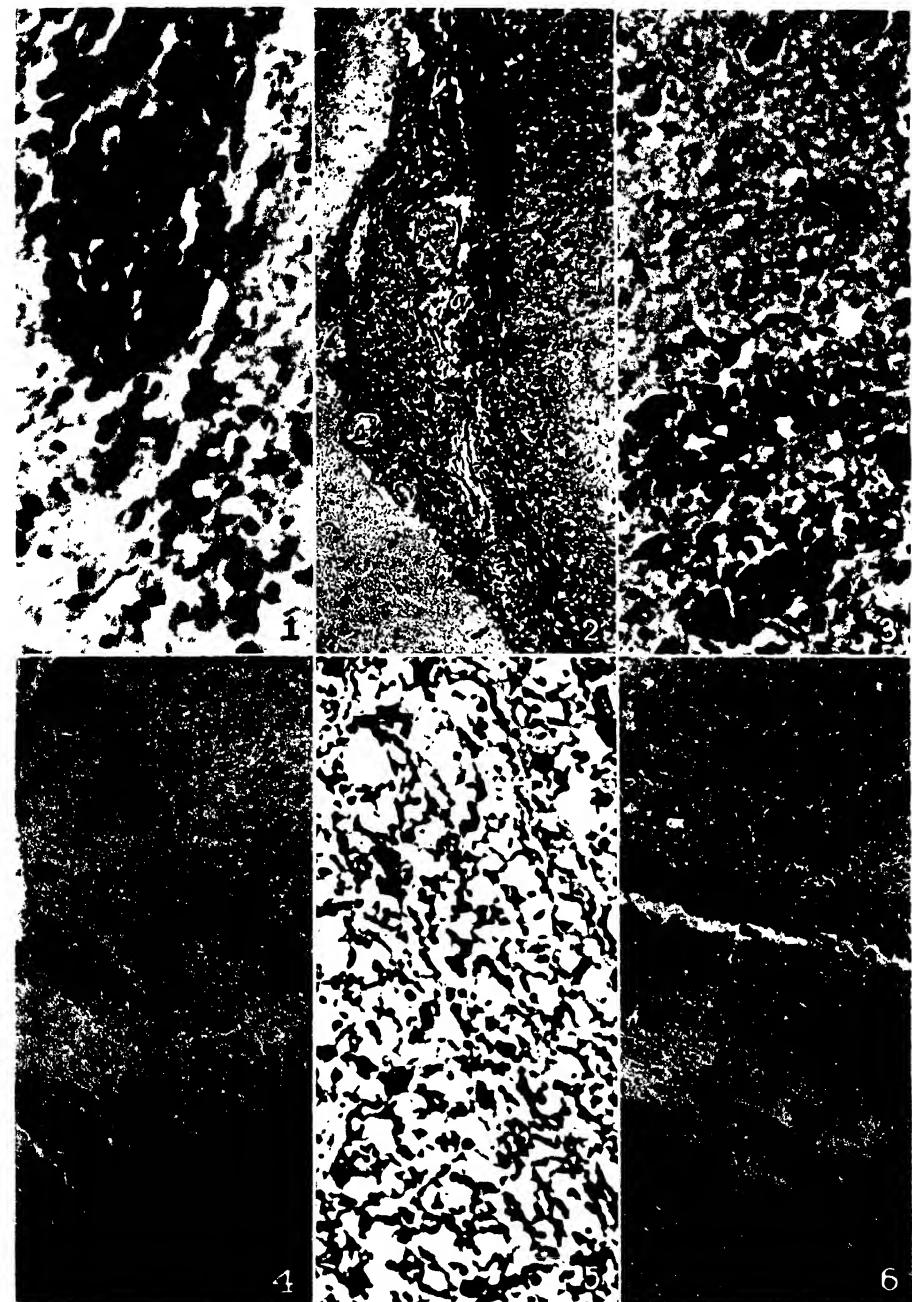
(Olitsky and Yager: Disseminated encephalomyelitis in mice)

FIG. 7. Mouse 3-5D, cervical cord. Area of necrosis without surrounding zone of inflammation and demyelination in white matter; also shrunken and degenerated neurones. Hematoxylin-eosin stain. × 207.

FIG. 8. Mouse 5-5B, thalamus. Glial infiltration of parenchyma (microglia and occasional gitter cells). Ranson's silver impregnation. × 394.

FIG. 9. Mouse 2-5B, thalamus. Nodular glial infiltration. Hematoxylin-eosin stain. × 406.

FIG. 10. Mouse 4-5E, neocortex. Vascular hyaline thrombus; also several vessels showing mural, perivascular, and extravascular infiltration. Hematoxylin-eosin stain. × 207.

FIG. 11. Mouse 7-5B, upper cervical cord, white matter. Massive lesion of vascular type, also diffuse glial infiltration of parenchyma and slight degree of demyelination at edge of lesion. Hematoxylin-eosin stain. × 165.

FIG. 12. Same mouse, cerebellum. Marked diffuse infiltration and vascular lesions in central white and granular laminae; Purkinje cells degenerated. Hematoxylin-eosin stain. × 287.

HEMAGGLUTINATION BY COLUMBIA SK, COLUMBIA MM, MENGO ENCEPHALOMYELITIS, AND ENCEPHALOMYO-CARDITIS VIRUSES: EXPERIMENTS WITH OTHER VIRUSES

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During the course of a study on hemagglutination by certain neurotropic viruses, a study suggested by the findings reported in the foregoing paper,[1] the writers' attention was directed to two reports. One[2] stated that the Lansing strain of poliomyelitis virus, and the other[3] that the Columbia SK (Col SK) and Columbia MM (Col MM) viruses agglutinated sheep erythrocytes, the agglutination being inhibited by specific antisera.

In our own work no hemagglutination by the Lansing strain was found; Hallauer[3] also reported failure. On the other hand, hemagglutination of sheep RBC by Col SK and Col MM viruses[3] was not only confirmed in the present investigation but a similar specific reaction was also obtained with Mengo encephalomyelitis (ME) and encephalomyocarditis (EMC) viruses.

This paper reports the results of these tests as well as attempts to disclose agglutination of sheep red cells and several additional types of erythrocytes by still other neurotropic viruses. Furthermore, there will be described an agglutinin for erythrocytes deriving from several species of animals present in suspensions of normal mouse brain, as well as an inhibitor of agglutination contained in the tissue suspensions and also in normal serum.

Hemagglutination of Sheep Erythrocytes by Col SK, Col MM, ME, and EMC Viruses. Dick and Taylor[4] have employed solutions of crystalline bovine plasma albumin (BPA) as a medium for preservation and for dilution of several viruses, among which were influenza, yellow fever, Lansing and ME. A 0.1 or 0.2% solution of the crystals in buffered saline solution, filtered through a Seitz apparatus generally sufficed for ordinary laboratory purposes, especially for dilution in titration tests.[4] It was also found in this laboratory that for the several viruses employed in the present study BPA was a satisfactory vehicle

[*] Lt. Colonel, V. C., U. S. Army.

[1] Lahelle, O., and Horsfall, F. L., Jr., Proc. Soc. Exp. Biol. and Med., 1949, 71, 713.

[2] Bremer, A., and Mutsaars, W., C. r. Soc. biol., 1948, 142, 1194.

[3] Hallauer, C., 4th Internat. Cong. Microbiol., July 20–26, 1947, Copenhagen, 1949, p. 257.

[4] Dick, G. W. A., and Taylor, R. M., J. Immunol., 1949, 62, 311.

and the advantages of utilizing a clear, nonagglutinating solution, which also preserved the titer of a virus in tests for hemagglutination, were apparent. Consequently virus-infected mouse brains, (20%) were suspended preferably in 0.1% BPA although saline solution or 10% rabbit serum could also be employed. Such suspensions were used in the fresh state; or if stored, were kept frozen in a mechanical, electrically operated freezer at −20 to −25°C and thawed just before use. Dilutions of virus for hemagglutination were made, however, with buffered saline solution, 0.85% NaCl, 0.02–0.05 M phosphate and pH 7.6.

The procedure of the test was as follows: The fresh or thawed viral suspension consisting of 20% brain tissue was centrifuged for clarification at 2,000 rpm for 5 minutes. 0.4 ml of the supernate was added to the first 2 of a series of 10 to 15 tubes. Buffered saline solution in equal amount was introduced into all tubes except the first to make a 2-fold dilution in a final volume of 0.4 ml in the second and successive tubes. To each virus dilution was added 0.4 ml of 0.5% washed sheep RBC suspended in buffered saline solution, thus securing final dilutions of virus of 1:10 to 1:5, 120, or higher, and of the erythrocytes in each tube, 0.25%. Cells were prepared from fresh bleedings and stored in modified Alsever's fluid (ACD);[4a] as such they could be kept for about 1 month in the ice box; after washing, however, not longer than 5 days. Hamster erythrocytes were prepared from fresh bleedings and were not satisfactorily stored; they were proved useless if kept in ACD for periods longer than 4 days. The tubes were shaken, kept for 60–120 minutes at 5°C and then read. It should be emphasized here that false positives, i.e., nonspecific reactions, easily obscured the results especially since mouse-brain suspensions were employed; the cause of this difficulty will soon be given. Hence tests should include a) a control on the virus suspension, namely, normal mouse brain suspension and b) antiserum to determine the specificity of hemagglutination. Generally the methods here described and the scale of reading agglutination, except for certain modifications, follow those already described.[1, 5] It should be stressed here also that even slight variations in technic sometimes brought about irregular results—a fact which applies also to the standard test.[5]

Table I shows the outcome of one of several similar experiments. The selective agglutination of sheep RBC by Col SK, Col MM, ME and EMC viruses is noted. It is not surprising to find such uniformity of reaction exhibited by the 4 viruses since it has already been found by Warren and Smadel[6] and by Dick[7]

[4a] Rapoport, S., J. Clin. Invest., 1947, 26, 591.

[5] Hirst, G. K., J. Exp. Med., 1942, 75, 49; Smadel, J. E., in Viral and Rickettsial Infections of Man, ed. T. M. Rivers, J. B. Lippincott Co., Philadelphia, 1948, Chap. 3, pp. 77–82.

[6] Warren, J., and Smadel, J. E., J. Immunol., in press.

[7] Dick, G. W. A., J. Immunol., in press.

that there is a close relationship among the members of this group as proved by the results of serological, immunological and other biological studies.

With these 4 viruses the hemagglutination was carried out best at 5°C for the reason that spontaneous elution or dispersion of virus from the erythrocytes occurred at room (23°C) or incubator (37°C) temperatures so that at the higher temperature little or no agglutination was visible. This reversibility of the agglutination by means of increasing the temperature could be carried out with the same materials for an indefinite number of times, or as long as sufficient virus survived the process to show its hemagglutinative capacity. In this respect, the present group of viruses behaved as did the GDVII virus.[1]

TABLE I

Hemagglutination of Sheep Cells by Columbia SK, Columbia MM, Mengo Encephalomyelitis and Encephalomyocarditis Viruses, Held for 120 Min. at 5°C.

	Virus or control materials	Reciprocal of final dilution of virus or of normal mouse brain							RBC of types other than sheep, plus virus (10 to 5,120 dils.)
		10	20	40	80	160	320	640 to 5,120	
Test	Col SK	4	4	2	±	0	0	0	0
	Col MM	4	4	2	0	0	0	0	0
	ME	4	3	3	3	2	±	0	0
	EMC	4	4	4	3	2	1	0	0
	West equine	0	0	0	0	0	0	0	0
	East "	0	0	0	0	0	0	0	0
	GDVII	0	0	0	0	0	0	0	+ with human-o cells only
Controls	Normal mouse brain	0	0	0	0	0	0	0	See text
		sheep cells							Cells alone
	Saline soln.	0							0
	BPA	0							0

Specificity of Hemagglutination. The next investigation related to the specificity of the hemagglutination for sheep erythrocytes by Col SK, Col MM, ME, and EMC viruses. The procedure followed the established principles[5] of a preliminary titration to determine the hemagglutination titer of the virus to be tested and of selection of a dilution of it which represented not less than 4 and not more than 8 agglutination units. 0.2 ml of this dilution of virus was added to 0.2 ml of antisera, then 0.4 cc of the cells—no preliminary period of incubation of antisera and virus was found necessary. The antisera used were prepared by injecting rabbits subcutaneously 3 times at weekly intervals with 1, 2, and 3 respectively, fresh or frozen virus-infected mouse brains. All serum whether immune or normal was inactivated by heating at 56°C

*for 30 minutes. The virus was kept constant at 4 or 8 units per tube and the
serum was diluted 2-fold beginning with 1:10, thus the final dilutions of serum*

TABLE II

*Hemagglutination-Inhibition by Antisera: Col SK, Col MM, ME and EMC Viruses and
Sheep Cells*

Virus	Antiserum*	Virus agg. units	Reciprocal of final dilution of serum							
			40	80	160	320	640	1,280	2,560	5,120
Col SK	Col SK	4	0	0	0	0	0	0	0	4
Col SK	NRS†	4	4	4	4	4	4	4	4	4
Col MM	Col MM	4	0	0	0	0	0	1	2	3‡
Col MM	NRS	4	4	4	4	4	4	4	4	4
ME	ME	8	1	0	0	0	0	0	3	4
ME	NRS	8	4	4	4	4	4	4	4	4
EMC	EMC	8	1	0	0	0	0	2	4	4
EMC	NRS	8	4	4	4	4	4	4	4	4
ME	WEE§	8	4	4	4	4	4	4	4	4
EMC	WEE	8	4	4	4	4	4	4	4	4

* All antisera were prepared by injecting rabbits repeatedly with mouse-brain virus.
† NRS = normal rabbit serum.
‡ The titration continued as follows: 10,240 read 3; 20,480 read 4.
§ Western equine encephalitis antiserum.

TABLE III

*Tests Showing Hemagglutination-Inhibition Titers of Antisera against Col SK Group of Viruses
and Certain of the Cross-Reactions*

Virus	Antiserum	Agglutination-Inhibition titer
Col SK	Col SK	1:2,560*
	Col MM	1:640
	ME	1:1,280
	EMC	1:1,280
ME	ME	1:2,560
	EMC	1:320
EMC	EMC	1:640
	Col SK	1:640

* The dilutions represent the highest dilution of antiserum preventing agglutination (Table II).

*became 1:40 to 1:20, 480, since 10 dilutions were usually tested. The test was
read after 60 minutes at 5°C. The reading of the hemagglutination-inhibition
followed standard methods.[5]*

Table II demonstrates one of the tests. It will be observed that not only did the antisera inhibit specifically the hemagglutination of sheep cells by the Col SK group of viruses but there was evidence of clear-cut cross-reactions among members of this group of viruses. Table III is presented to demonstrate the results of a test on inhibition of hemagglutination, the titer of antisera and certain cross-reactions among the members of the Col SK group of viruses.

It is concluded therefore that the Col SK, Col MM, ME and EMC viruses agglutinate sheep RBC specifically; they exhibit cross-agglutination inhibition among the individuals of the group and since other neurotropic viruses do not agglutinate sheep cells, as will be shown immediately, these 4 infective agents can be looked upon as having a generic relationship and a common antigenicity. Thus support is given to the findings of Warren and Smadel[6] and of Dick[7] who produced solid evidence from a wholly different approach to the problem of interrelationship of the 4 agents.

Since the hemagglutination is characteristic, it can apparently be utilized for the identification of the viruses of the Col SK group and for measurement of the antibody content of antisera against any of the 4 agents. Finally, since all of the individual members of the group agglutinate sheep RBC in the cold, elute or disperse spontaneously and rapidly at moderate elevations of temperature, and when freed from the erythrocytes are as active as they were originally, hemagglutination may be useful for purposes of adsorption, without inactivation, of the active agents, just as can be done with the GDVII virus.[1]

Agglutination Tests with Other Neurotropic Viruses and a Variety of RBC. A wide variety of erythrocytes other than those of sheep, namely, human O, chick, horse, hamster, dog, cat and guinea pig, were tested for agglutination by the 4 viruses of the Col SK group; the tests failed. In addition, the various erythrocytes just mentioned, including sheep cells, were tested for agglutinability by numerous neurotropic viruses. The viruses employed were:

Eastern equine encephalitis	Theiler (FA strain)
Western equine encephalitis	Theiler (TO strain)
Venezuelan equine encephalitis	Poliomyelitis (Lansing strain)
Japanese B encephalitis	Poliomyelitis (MEF1 strain)
St. Louis encephalitis	West Nile
Russian Far East encephalitis	Rabies
Vesicular stomatitis (New Jersey strain)	Lymphocytic choriomeningitis
Vesicular stomatitis (Indiana strain)	Herpes simplex
	Louping ill

The results of over 100 experiments can be summarized by stating that no specific clumping of any of the types of erythrocytes by any one of the viruses mentioned was detectable. Now and again agglutination was seen but further study revealed it to be nonspecific, most often owing to the physical or particulate condition of the mouse-brain suspension. It was shown that a) normal

mouse-brain suspensions produced similar hemagglutination; b) centrifugation to a degree which clarified the suspension but did not sediment the virus from the supernate served to abolish the agglutinative capacity of the supernate, c) antisera failed to inhibit the reaction, d) the reaction was not reversible, e) the aggregations formed did not resemble the "soft", clinging, fine agglomerations as was seen in the hemagglutination by Col SK group of viruses just described. On the contrary, the aggregates were usually "hard", coarse, irregularly sized and shaped, and sometimes were surrounded by a narrow zone of slight hemolysis.

In view of the fact that GDVII virus exhibits agglutination only of human-O RBC[1] and the Col SK group of viruses only of sheep cells, one may well question the meaning of the negative results obtained with the other neurotropic viruses and the kinds of erythrocytes used in the present investigation. Since agglutination is so selective with respect to cells used, it will not be surprising to find one or another of these viruses yielding positive results with erythrocytes deriving from species not as yet tested.

Agglutinative Capacity of Normal Mouse-Brain Suspensions for Dog, Cat and Guinea Pig RBC. During the course of the present study, it was noted that suspensions of normal mouse brain agglutinated to a low degree, dog, cat, and guinea pig RBC. The hemagglutination titers were generally 1:10, less often 1:20, rarely 1:40, and of 15 samples in one instance only, 1:320. This reaction was observed after 60 minutes at 5°C, but the maximum titer was reached, however, at room temperature. The reaction was not reversible: there was no visible phenomenon similar to that of spontaneous elution. Moreover, antisera prepared by immunizing rabbits against normal mouse brain had no inhibitory effect on the agglutinative power of the normal mouse brain. The agglutination thus produced revealed, therefore, characteristics unlike those of the viruses of the Col SK group. It is clear, however, that since neurotropic viruses are often employed in the form of infected mouse brain, the occurrence of this nonspecific hemagglutination should be reckoned with in experimental studies.

Presence of an Agglutinin and an Agglutination-Inhibitor for Hamster Cells in Normal Mouse Brain. It was also disclosed during the course of the present investigation that there exists in normal mouse brain, and therefore in virus suspensions prepared with infected mouse brain, an agglutinin as well as an agglutination-inhibitor (HI) for hamster erythrocytes, both being present in the same suspensions at the same time.

The agglutinin for hamster cells present in normal and virus-infected mouse brain became evident at ice box or room temperature, maximal after 2 hours' incubation. The agglutinated masses of hamster cells which formed were more minute, delicate and evenly dispersed along the sides of the test tube than were the aggregates formed by the Col SK group of viruses. Specific antisera did not

inhibit the hamster-erythrocyte agglutination to any greater extent than did normal serum of the same species. As will be shown later all sera contained a nonspecific inhibitor. It is therefore plain that this agglutinin has characteristics which differ from those shown by the Col SK group of viruses in the presence of sheep cells. The hamster-RBC type agglutination also differs from that observed when normal or infected mouse brain reacted with dog, cat or guinea pig erythrocytes.

The HI exerted its influence in dilutions of 1:40 to 1:160, rarely (3 of 18 titrations) as high as 1:640. After the HI was diluted out hemagglutination was then visible and showed itself in dilutions usually up to 1:80,000 and sometimes higher (Table IV). The HI was active both at ice box and room

TABLE IV

Presence of Hemagglutination-Inhibitor and Agglutination of Hamster RBC by Normal Mouse Brain (NMB) and Virus-Infected Mouse Brain (120 min. at 5°C)

Material used	Dilution of brain tissue or of antiserum*											
	40	80	160	320	640	1,280	2,560	5,120	10,240	20,480	40,960	81,920
NMB......................	0	0	0	0	0	3	4	4	4	4	4	4
NMB + its antiserum........	0	0	0	0	0	0	2	2	3	3	—	—
NMB + NRS*..............	0	0	0	0	±	1	4	4	4	4	—	—
West equine virus...........	0	0	0	1	3	4	4	4	4	4	4	2
West equine virus + its antiserum....................	0	0	0	1	2	4	4	4	4	—	—	—
NMB heated, 56°C, 10 min.....	4	4	4	4	4	4	4	4	4	4	4	4
NMB heated, 56°C, 30 min.....	4	4	4	4	4	4	4	4	4	4	2	0

* NRS = Normal rabbit serum; in an HI test, 8 units of normal or virus-infected mouse brain was used in each tube; reciprocal of final dilution is given.

temperatures and the maximum titers were noted after 2 hours' incubation. The inhibitor was thermolabile and could be inactivated by heating mouse-brain suspensions at 56°C for 10 to 30 minutes at which temperature the agglutinin was not affected (Table IV). Sodium citrate 2.5% did not neutralize its effect as it does in the case of PVM and other viruses.[8] It could not be sedimented out of a suspension of mouse brain at 3,000 rpm for 10 minutes and rabbit antisera prepared by repeated injection of normal or virus-infected mouse brain failed to reduce its titer of activity.

Nonspecific HI in Certain Normal Serum and Antisera. Antisera prepared in rabbits, guinea pigs, or monkeys, and the respective normal sera of these species possessed the capacity to inhibit hamster-cell agglutination by normal or virus-infected mouse-brain suspensions. Thus the nonspecific inhibition

[8] Ginsberg, H. S., personal communication.

could be demonstrated in dilutions of sera up to 1:1,280 (Table IV). When normal guinea pig or rabbit serum was heated at 65°C for 30 minutes the contained nonspecific inhibitor was not thereby inactivated. It has been previously reported[8, 9] that such treatment of serum can inactivate the inherent nonspecific inhibitor of hemagglutination by other viruses.

Summary of Nonspecific Agglutinins and Inhibitors. In sharp contrast to the clearly defined, specific agglutination of sheep red cells by the Col SK group of viruses on one side, and the failure of many other neurotropic viruses to show hemagglutination on the other, is the existence of the following nonspecific elements:

a) An agglutinin for dog, cat and guinea pig erythrocytes is contained in normal, or virus-infected mouse brain.

b) An agglutinin for hamster red cells is present in normal or virus-infected mouse brain.

c) A thermolabile inhibitor of agglutination of hamster RBC exists concomitantly with the agglutinin for the hamster cells just mentioned, in normal or virus-infected mouse brain.

d) A thermostable inhibitor is present in normal monkey, rabbit and guinea pig serum, consequently in antisera as well, which prevents agglutination of hamster cells.

The nonspecific reactions just described require further study for the identification of the agglutinins and the inhibitors present in mouse-brain suspensions and in normal serum. For the moment, the use of erythrocytes deriving from hamsters, dogs, cats and guinea pigs for agglutination by neurotropic viruses in the form of mouse-brain suspensions would appear to require caution. Since all the standard hemagglutination tests, now routine in laboratory practice, require careful control of the variables employed, similar precautions are needed as well for the Col SK, Col MM, ME, and EMC viruses.

Conclusions. Evidence has been brought forward to indicate that Columbia SK, Columbia MM, Mengo encephalomyelitis and encephalomyocarditis viruses agglutinate sheep red cells. It is therefore possible to identify these viruses by means of hemagglutination and to measure the antibody content of antisera. Since the viruses show characteristic spontaneous elution or dispersion from the erythrocytes, the method can be used for purposes of selective adsorption of the viruses without loss of their hemagglutinative activity. Moreover, the uniformity of the hemagglutination reaction shown by the 4 viruses and the cross-inhibition that exists among them supports the findings of Warren and Smadel[6] and of Dick[7] that these viruses are similar in many respects and are of the same group.

[9] Ginsberg, H. S., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1949, **89**, 37.

Seventeen other neurotropic viruses were tested for their capacity to agglutinate characteristically erythrocytes deriving from sheep, man (group 0), chicken, horse, hamster, dog, cat and guinea pig; these tests failed.

Another phenomenon that was observed is the nonspecific agglutination of dog, cat and guinea pig erythrocytes by normal or virus-infected mouse brains. With respect to agglutination of hamster cells, an inhibitor of agglutination is present not only in suspensions of normal mouse brain but also in normal serum and antiserum against the neurotropic viruses.

The fact that neurotropic viruses are often used in the form of mouse-brain suspensions renders it important therefore for investigators to use proper controls for the variables of the test and, in addition, to identify positive reactions by specific means.

HEMAGGLUTINATION AND COMPLEMENT FIXATION WITH TYPE I AND II ALBANY STRAINS OF COXSACKIE VIRUS*

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Dalldorf and Sickles(1) isolated a virus from the feces of patients having symptoms resembling those of abortive or even paralytic poliomyelitis. This active agent, characterized by pathogenicity for suckling but not for adult mice, was later named Coxsackie virus.(2) Melnick, Shaw, and Curnen(3) recovered several similar viruses some of which they found to be distinct by the neutralization test. Dalldorf(4) also determined such specificity for his strains, here designated as Type I and Type II Albany strains. The difficulties of working with infant mice are considerable; we were thus led to a study of a possible *in vitro* reaction that might be applicable for the identification of the virus, namely, hemagglutination or complement fixation. The present report concerns itself with the results of studies of the two reactions employing 2 of the Albany strains, Type I (T.T.) and Type II (Fleetwood), generously presented to us by Dr. Dalldorf.

Hemagglutination Tests. Agglutination tests were carried out in a manner already described,(5) except that the saline solution used for dilution of materials was buffered with 0.02M, instead of 0.05M phosphate. Human group O, sheep, chick, and guinea pig RBC were employed and the two different strains, Type I and II, of the Coxsackie virus. In addition, two forms of each strain were studied; suspensions of infected brain and of limbs, derived from infant mice which had reacted after their injection intraperitoneally of either active brain or limb virus.(6) The tubes containing materials were held at 5, 23, and 37°C and read at intervals up to 2 hours. As an indicator of the possibility of the materials yielding positive reactions, a test was set up with sheep

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1. Dalldorf, G., and Sickles, G. M., *Science*, 1948, v108, 61.

2. Gifford, R., and Dalldorf, G., Proc. Soc. Exp. Biol. and Med., 1949, v71, 589.

3. Melnick, J. L., Shaw, E. W., and Curnen, E. C., Proc. Soc. Exp. Biol. and Med., 1949, v71, 344.

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5. Olitsky, P. K., and Yager, R. H., Proc. Soc. Exp. Biol. and Med., 1949, v71, 719.

6. Dalldorf, G., Sickles, G. M., Plager, H., and Gifford, R., *J. Exp. Med.*, 1949, v89, 567.

cells and MM virus; definite agglutination was seen in 1:160 dilution of the virus after 1 hour at 5°C.(5) The results of all the hemagglutination tests with the 2 strains of Coxsackie virus were, however, negative. It should be stressed that suspensions of normal mouse brain, especially of fresh brain, if not properly centrifuged, show agglutination of sheep RBC, the degree of hemagglutination depending on the particles in the supernate.

Complement-fixation tests. Complement-fixation tests have been carried out with the same Albany strains using mouse immune serum and antigens derived from infected brain or limb tissue of suckling mice, Swiss-W strain.[1]

Immune Serum. Antisera were prepared in 3- to 4-month-old Swiss-W mice by giving each of them, intraperitoneally, 0.5 ml of mouse-brain virus, 10^{-1} dilution, on the 1st and 5th day; they were bled on the 15th day. These animals

TABLE I

Showing Complement-fixation Tests with Albany Strains of Coxsackie Virus

Antigen	Serum					
	Type I brain	Type I limb	Type II brain	Type II limb	Normal	Western equine
Type I limb....................	0	1/16	0	0	0	0
Type I brain....................	0	0	0	0	0	0
Type II limb....................	0	0	1/2	1/32	0	0
Normal limb....................	0	0	0	0	0	0
Western equine brain...........	0	0	0	0	0	1/64

Fractions represent the highest dilution of serum showing 2+ or better complement fixation; in the test, serum was diluted 2-fold beginning with 1:2 dilution.

were then injected intraperitoneally with limb virus, each with 0.5 ml of 10^{-1} dilution, on the 19th and 25th day; they were bled again on the 35th day. In all 20 mice were so treated with each type of virus.

Antigen for test. Antigens for complement-fixation tests were prepared following a method previously described (acetone-ether extracted antigens).(7) In the present study, 2- to 11-day-old Swiss mice were given .05 ml of 10^{-1} suspension of infected brain or limb tissue intraperitoneally. When definitely ill,[1] usually on the 3rd day, brain and limb tissue, the skin being removed from the latter, was collected separately and prepared(7) as antigens. It should be stated here that the titer of virus in limb tissue derived from 11-day-old mice was greater than $10^{-7.5}$, confirming the observations of Gifford and Dalldorf.(2) Extraction of the limb tissue with acetone-ether was found to be more satisfactory than the method of suspension in saline solution followed by centrifu-

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[1] In experiments with animals deep ether anesthesia was employed.

7. Casals, J., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 339.

gation at 12,000 rpm. For control material, brain and limb tissue was collected from apparently normal Swiss mice of the same age. The complement-fixation test was performed in the manner previously described.(8)

Experimental. Table I illustrates one of the tests. A specific fixation was obtained with both types of Coxsackie virus, used as limb-tissue antigen, without any demonstrable sign of cross reaction. Moreover, antigens made up of brain-tissue virus were not effective. Indeed, following the injection of brain virus into mice, their sera were either negative or of a low titer; whereas after immunization with limb-tissue virus a high titer of fixing antibody was obtained. It would appear that the limb-tissue virus is a highly potent antigen. Finally, no reaction took place between Coxsackie-virus sera or antigens and unrelated virus or apparently normal materials.

Summary. Two Albany strains of Coxsackie virus, Type I (T.T.) and Type II (Fleetwood) were found to be negative for hemagglutination when human O, sheep, chick, and guinea pig erythrocytes were used. On the other hand, the viruses showed distinct positive specific complement fixation without any cross reaction between them. Antigens prepared from infected brain tissue were inactive; those from infected limb tissue were highly active. Tests with human sera and limb-tissue antigens are left for further study.

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SEQUENCES IN THE FORMATION OF CLOTS FROM PURIFIED BOVINE FIBRINOGEN AND THROMBIN: A STUDY WITH THE ELECTRON MICROSCOPE*

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Plates 21 to 23

In recent years studies of the clotting of blood have resulted in the chemical identification and isolation of some of the factors involved, and these studies together with clinical observations have contributed to an as yet incomplete description of the phenomenon. Investigations on both the morphological and chemical aspects of the problem have been greatly facilitated by the quantities of relatively pure materials made available through methods developed by Cohn and his associates (1–4).

In contrast to the many physiological and chemical contributions to our understanding of the clotting process, there have been few recently that could be classed as morphological. Howell (5), in 1914, made use of the ultramicroscope to observe the conversion of fibrinogen to fibrin. He described the end result as "a formation of a meshwork of beautiful needles," and suggested that the matter of greatest interest was to attempt to follow the actual process of formation of these needles. However, he commented that it was in the background of particles beyond the vision of the ultramicroscope that the "needles" or "crystals" of fibrin took their form.

The resolving power of the electron microscope allows observations to be made in this previously inaccessible size range. In an earlier report (6) the authors described the fine structure of clots formed from purified bovine fibrinogen and thrombin. The techniques used in that study seemed also to be applicable to the study of sequences in the conversion of fibrinogen to fibrin by the action of thrombin.

Materials and Methods

Inasmuch as the present series of experiments required certain alterations in the previously described techniques, repetition and amplification of some of the details seem necessary.

* Presented at the 1948 meetings of the International Society of Hematologists held in Buffalo, New York.

‡ Affiliated with Columbia University.

The fibrinogen and thrombin preparations used in these experiments were generously provided by the Chemical Research and Development Department of Armour and Company, Chicago. The fibrinogen (lot C-185A) contained 79 per cent clottable protein and was furnished in the form of a dried powder of which approximately 40 per cent by weight was sodium citrate. The thrombin had a clotting activity of 27 units per mg. of total weight. These substances were dissolved in buffered physiological saline or in Tyrode's solution as indicated in the figure legends. Determinations of the pH were made with the glass electrode. The solutions were allowed to react at room temperature (22.0–23.0°C.).

Conventional glass microscopic slides (1 inch × 3 inches) were split longitudinally and coated with a thin film of formvar by immersing them in a 0.15 per cent solution of this material in ethylene dichloride and allowing the solvent to evaporate. The resulting film was approximately 200 Å in thickness.

A clotting mixture was prepared by the addition of thrombin in buffered saline solution at a concentration of 10 units per cc. to the fibrinogen in similarly buffered solutions at a concentration of 0.06 per cent. To 4.5 cc. of the fibrinogen solution in a short test tube, 0.5 cc. of the thrombin solution was added and mixed rapidly by pipetting. The coated slide was immersed in the clotting mixture, then removed and placed horizontally over water in a covered Petri dish.

After various time intervals arbitrarily selected the polymerization process was interrupted by the exposure of the thin film of clotting mixture to vapors of OsO_4 or to the action of a 0.2 per cent solution of phosphotungstic acid. The rapid penetration of the clot film by these fixatives gave reproducible results, apparently satisfactory for the experiments under consideration. After fixation for 10 to 15 minutes, the slide was flooded with distilled water and small portions of the clot-covered film of formvar were peeled away from the glass and mounted on the conventional 3 mm. discs of wire cloth used to support objects for electron microscopy. The preparations were then drained, dried, and examined. Many were later subjected to "shadowing" with gold or chromium or uranium in order to bring out details not readily demonstrated in the preparations not so treated (7). In some instances the preparations after fixation were dried for 1 to 2 hours over P_2O_5 before the screens were made.

The formed clot in the test tube was removed and compressed to obtain free fluid for pH determinations, as a first approximation of the measurement of the pH of the forming clot on the microscope slide.

In order to complete the series, slides were also dipped into the solution of fibrinogen (without thrombin), fixed in vapors of OsO_4, washed, and mounted. These were supplemented by preparations in which the fibrinogen and fibrinogen-thrombin mixtures were nebulized onto coated screens after fixation (Fig. 1).[1]

Because of the significant variations in clot structure induced by changes in pH as previously reported (6, 8), these experiments in clot formation were carried out over as narrow a range of hydrogen ion concentration as was practicable. The pH chosen was that at which polymerization was known to result in well defined fibers.

Observations

The following description of the clotting process is an attempted integration of impressions gained from the study of many micrographic fields in each preparation. It must be stressed that clotting is a continuous process and that

[1] The authors are indebted to Dr. Robley C. Williams of the University of Michigan for suggesting nebulization as a means of providing adjacent surfaces of supporting membrane and material being studied.

until the process was completed various stages could be found at each of the arbitrary time intervals selected.

Fibrinogen, placed on the supporting formvar film without being exposed to the action of thrombin, appeared as a finely particulate film in which there was random arrangement of densities and no clear evidence of fibrillar structure (Figs. 1 and 2).

The first evidence of the clotting process was seen in the films prepared from the clotting mixture 15 seconds after mixing the fibrinogen and thrombin solutions. The particulate densities showed arrangement into short chains (Figs. 3, 4, and also in background of other figures). In scattered areas there were small needle-like structures which apparently had resulted from the lateral association of several of these short chains of particles, or protofibrils (Figs. 4 to 7).

As the clotting process progressed, the needle-like structures increased in length and diameter apparently by association with others of similar size and by the continued addition of more fibrinogen. In any preparation in which clotting had been allowed to proceed for about 30 seconds it was possible to find these slender spindle-shaped (tactoid-like) fibrils in a continuous series of sizes (Fig. 6).

At these and later stages the larger fibrils (referred to below as unit fibers) demonstrated a similar tendency to aggregate. They seemed to be influenced to align themselves in association with other formed fibrils lying in random arrangement in various planes. This alignment was by lateral association along the long axes of the fibers and particularly by the lateral association of their tapering ends (Figs. 5 to 7). The number of such fibers coming together in lateral association to form the compound fibers of the developing clot increased with time. It was apparent that portions of some of these fibers lay in association with more than one compound fiber so that interconnections were formed giving the appearance of branching of the resultant strands (Figs. 7 to 9). This of course provided as well tridimensional continuity to the framework of the clot. A striking feature of the formed bundles was that the unit fibers retained their identity within the strands (Figs. 8 and 9). A possible significance of this is discussed subsequently.

The appearance of the completed clot was described in the previous report (6). The striking regularity of the coinciding densities or cross-striations of the crystal-like fibers and the fibers formed of fibrils in lateral association was again obvious in the preparations made for these present experiments (Fig. 9).

DISCUSSION

The formation of crystal-like structures was a striking feature of the clotting process as observed under the conditions of these experiments and was described by Howell and others using comparable systems for study by means

of the ultramicroscope. In addition, the identity of these crystal-like fibers was retained in the compound fiber strands after the lateral association of the unit fibers. It does not seem to us, therefore, that the tridimensional network of fibers which is the mature clot can be described as a continuous three dimensional polymerization. The evidence from light and electron microscopy suggests rather that the crystal-like unit fibers are formed first and as a final stage these associate to form the rigid clot.

It is of interest to note that Ferry and Morrison, studying the clotting time of similar systems, observed that the effect of pH was opposite to what might have been expected. Increasing pH increased the fineness of the clot and if this were due to a change in fibrinogen interaction, a prolongation of clotting time was to have been expected; actually the time was shortened (8). Our own observations indicate that in clots formed at an alkaline pH the tendency for lateral association of unit fibers or crystals was markedly decreased and the greater number of small fibers that were formed became associated as such to form the fine transparent clot (6). As a result of this the clot more nearly resembled the meshwork that one might expect from polymerization alone (8) without the formation of discrete crystals and then subsequent alignment into fibers. Hence, this type of clot might be more simply and more rapidly formed than the "coarse" clot (pH 6.5) in the formation of which a lateral association of unit fibers to form larger needle-like crystals is a striking characteristic.

A morphological description of the clotting process invites speculation as to changes at the molecular level. The available data compiled from physical constants measured on some preparations of human fibrinogen have been presented by Edsall, Foster, and Scheinberg (9). Nanninga (10) has studied bovine fibrinogen prepared by salting out with ammonium sulfate. He obtained a molecular weight of 441,000 by measurements of osmotic pressures and a value from viscosity studies indicating an axial ratio (prolate ellipsoid) of approximately 20 to 1, from which he concluded that the molecular length was 725 Å. This deduced model is remarkably close to that proposed by Edsall and his associates. However, as Edsall pointed out, the discussion and deductions presupposed that the fibrinogen molecule is rod-shaped. He added that a model consisting of a disk-shaped, oblate ellipsoid reproduces all the experimental data at least as well as any of the prolate ellipsoidal molecules. This disk-shaped model would only be about 9.8 Å thick at the center with a diameter of 343 Å. Edsall considered such a model to be highly dubious. However, he felt it conceivable "that the fibrinogen molecule might be built up of a peptide chain coiled around itself in a single plane in such a manner as to form a flat disk" and speculated that the initial "action of thrombin on fibrinogen would then presumably be the loosening of the attachments holding the

different portions of the disc together, so that the molecule could uncoil into a long, thread-like structure" (9).

Studies by Bailey, Astbury, and Rudall (11), using the technique of x-ray diffraction, indicated that fibrinogen and fibrin belonged to the keratin-myosin group of fibrous proteins. Further, they concluded from their observations that fibrin is no other than an insoluble modification of fibrinogen without any fundamental change in molecular plan, though it is in a higher state of aggregation, as is evidenced by its greater internal cohesion and the ease of transformation from the α-keratin kind of x-ray diffraction pattern to the β-keratin kind by stretching.

Attempts at visualization of unclotted fibrinogen by various electron-micrographic techniques with which the authors are acquainted have thus far failed to show elongated fibrillar structures, although one might expect resolution of structures of the order of magnitude of the proposed rod-shaped molecule (700 Å × 30 Å) especially in metal-shadowed preparations. Instead, only single particulate bodies are observable[2] (Figs. 1 and 2).

As has been previously described (6), a striking feature of all the clots studied is cross-striation of the fibers (Figs. 9 and 11). The periodicity of these striae is quite constant throughout, approximately 250 Å. Similar periodically recurring densities are illustrated in the forming crystal-like structures described in the present experiments.

In an attempt to correlate the observations derived from the studies of double refraction of flow, specific viscosity, sedimentation constant, and molecular weight of fibrinogen in solution, with the observations made on the x-ray diffraction patterns of fibrinogen and fibrin, and with our own observations made on the material prepared for study by means of the electron microscope, we propose the following speculations:

The initial conversion of fibrinogen molecules to protofibrils of fibrin results from the thrombin-catalyzed interaction between disk-shaped molecules which may become aligned edge to edge as well as face to face (Fig. 12).

The resulting fibril would show periodically recurring densities in electron micrographs of either the faces or the edges of the disks presented (see protofibrils in Figs. 3 and 10), if one makes the assumption that the regions of greatest opacity to the electron beam correspond to the greatest volume of protein material presented to that beam. The dehydration of the fibrin prepared for study in the vacuum of the electron microscope would be expected to reduce somewhat the dimensions of the individual molecules. The association of several such protofibrils, as illustrated in Fig. 13, and the simultaneous addition

[2] In addition to the procedures described in the text, fibrinogen solutions have been examined after rapid freeze-dry treatment and also after replica procedures designed to eliminate the confusing image of the formvar or collodion film.

of more fibrinogen molecules would yield the small needle-like crystals or unit fibers characteristic of the earlier stages of clotting (Figs. 4 and 5).

The observations we have presented are morphological. No conclusions are drawn as to the chemical changes in fibrinogen, presumably intermolecular, instituted by the action of thrombin, or the nature of the bonding by which the units are held together. It would seem that the forces responsible for the formation of protofibrils as well as the precise lateral association of these and the much larger crystal-like unit fibers must operate over relatively large distances when one takes into account the extreme dilutions at which clotting can be effected[3] and the pronounced asymmetry of the structures involved.[4]

SUMMARY

The observed sequences in the formation of clots from purified bovine fibrinogen and thrombin are described. Under the conditions of these experiments, it appears that fibrinogen molecules are polymerized by the action of thrombin to form needle-shaped, crystal-like protofibrils which then become aligned into fiber strands by lateral association. The integrity of the unit fibrils is maintained within the strand.

A model of the fibrinogen molecule is proposed which may satisfy the reported physical constants, data from x-ray diffraction studies, and observations made upon electron micrographs.

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[3] Ferry and Morrison (8) report that concentrations of fibrinogen as low as 0.002 *g* per cent will yield a rigid clot.

[4] Since the submission of this report for publication, the observations of Cecil E. Hall have been published in *The Journal of Biological Chemistry*, June, 1949. Inasmuch as this investigator has used techniques of preparation which differ somewhat from those described in this report, it is difficult to make direct comparisons. It seems possible to us that the fibrillar structures illustrated and described by Hall represent fibrinogen partially associated because his illustrations closely resemble the background fibrillar material observed by us in fibrinogen-thrombin preparations but not in fibrinogen alone. It deserves mention that in the preparation of electron microscope specimens of this sort, adsorptive, denaturing, and drying effects are always present.

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EXPLANATION OF PLATES

PLATE 21

FIG. 1. Electron micrograph of the margin of a dried droplet of fibrinogen solution, taken to show particulate non-fibrillar character of the dried material. A 0.06 per cent solution of fibrinogen in Tyrode's solution was mixed with 5 volumes of 0.2 per cent OsO_4 solution in H_2O and nebulized onto formvar-coated slides. After the preparation had dried it was immersed in water and small sections of the formvar were peeled off the glass and mounted on screens. These were shadowed with gold at an angle of 12°.

The particulates of the droplet on the left are easily distinguished from the granular appearance of the formvar surface at the right. The fibrinogen particulates very considerably in size; the most commonly occurring size is about 200 Å. $\times$ 30,500.

FIG. 2. Electron micrograph of fibrinogen alone. A formvar-coated slide was immersed in 0.06 per cent solution of fibrinogen (pH 7.0) and after 15 seconds the slide was withdrawn and flooded with a 0.2 per cent solution of phosphotungstic acid. Fixation was continued for 10 minutes. The preparation was then immersed in water and portions of the protein-covered film were mounted on screens, drained, and dried. It was subsequently shadowed with gold at an angle of 10°. Note the particulate character of the material and the unorganized distribution of the particles. $\times$ 28,500.

FIG. 3. Electron micrograph of fibrinogen-thrombin mixture taken on a screen prepared by the same procedure as that used for the material shown in Fig. 2. The starting material in this case was a mixture of 9 parts of a 0.06 per cent solution of fibrinogen in Tyrode's solution (pH 7.1) and 1 part of a solution of thrombin (10 units/ml.) in the same fluid. As soon as the two components had been mixed the coated slide was immersed in the mixture and at the end of 15 seconds, it was withdrawn and flooded with a 0.2 per cent solution of phosphotungsticacid. It can be seen that, under the influence of the thrombin, the particulate material is organized into strands or protofibrils. These obviously vary some in width, but the more commonly occurring, fundamental strand measures between 200 and 230 Å. $\times$ 28,500.

FIG. 4. Electron micrograph showing the earliest development of unit fibers, apparently from the union of several protofibrils. The preparation was made in the same way as that examined for Fig. 3 except that clotting (at pH 6.7) was not interrupted until after 25 seconds. Fixation was performed with 0.2 per cent phosphotungstic acid in water. The background shows the development of protofibrils characteristic of all fibrinogen-thrombin preparations. $\times$ 23,000.
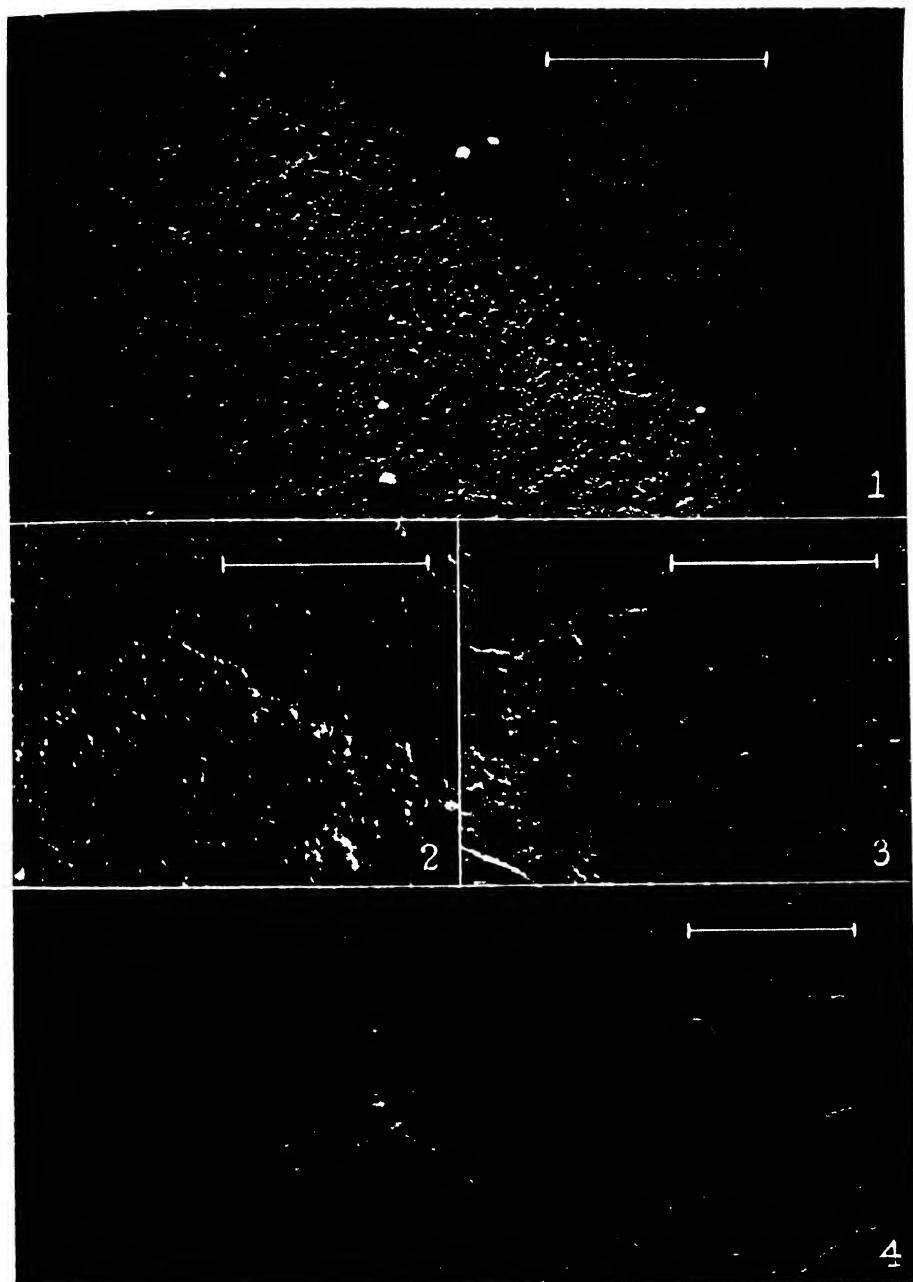
(Porter and Hawn: Formation of clots from fibrinogen and thrombin)

PLATE 22

FIG. 5. Electron micrograph of an unshadowed preparation of clotting mixture in which the clotting process (at pH 6.7) was interrupted after 30 seconds. Material was fixed and stained with 0.2 per cent phosphotungstic acid. The unit fibers in the center of the picture appear to be developing from an aggregation of protofibrils. The banded or striated structure characteristic of the larger fibrin strands can be detected also in a few of these small unit fibers. × 20,500.

FIG. 6. Electron micrograph to show needle-like crystals of fibrin that may develop in preparations within 60 seconds of mixing the fibrinogen and thrombin. The clotting mixture consisted of 9 parts of a 0.06 per cent fibrinogen solution in Tyrode's (pH 6.3) and 1 part of a thrombin solution containing 10 units per ml. A coated slide was immersed in the mixture for 15 seconds, removed, and placed in a horizontal position for 45 seconds. The clotting process was then stopped with vapors of OsO_4 which were allowed to act for 10 minutes. The fixed preparation was then dried. It was later immersed in H_2O and made up into several screens. One gains from the micrograph the impression that the needles or unit fibers are lining up in parallel array to form a major strand of fibrin. The unit fibers shown here vary in width from 50 mμ to 150 mμ and in length from 0.6 micron to 2.5 microns. × 9,500.

FIG. 7. Electron micrograph showing association of small unit fibers to form a continuous meshwork; clotting mixture same as used in other preparations (pH 7.0). Clotting was permitted to continue for 30 seconds before fixation with phosphotungstic acid. × 13,500.

FIG. 8. Electron micrograph designed to show character and arrangement of fibers after a longer period of clotting. The clotting mixture was the same as for the preparation shown in Fig. 6. Clotting (at pH 6.3) was allowed to continue for 150 seconds. The preparation was fixed with OsO_4 for 10 minutes, dried, and subsequently made into screens. It can be seen that the strands are compound in structure; *i.e.*, made up of several unit fibers or needle-like crystals of the sort shown less closely associated in Fig. 6. Some of the unit fibers form connections between the larger strands, having one end attached to one fiber and the other end to another fiber. It is characteristic for unit fibers formed at this lower pH to be relatively straight and apparently rigid. The striated structure of the fibers (which is shown in Figs. 9 and 11) and the protofibrils of the background are hidden by the relatively large amount of unpolymerized protein (non-fibrous) present when the preparation was dried. Preparations fixed with either OsO_4 or phosphotungstic acid and made into screens without previous drying (Figs. 4, 7, and 11) are cleaner and reveal more of the fine structure. × 12,500.
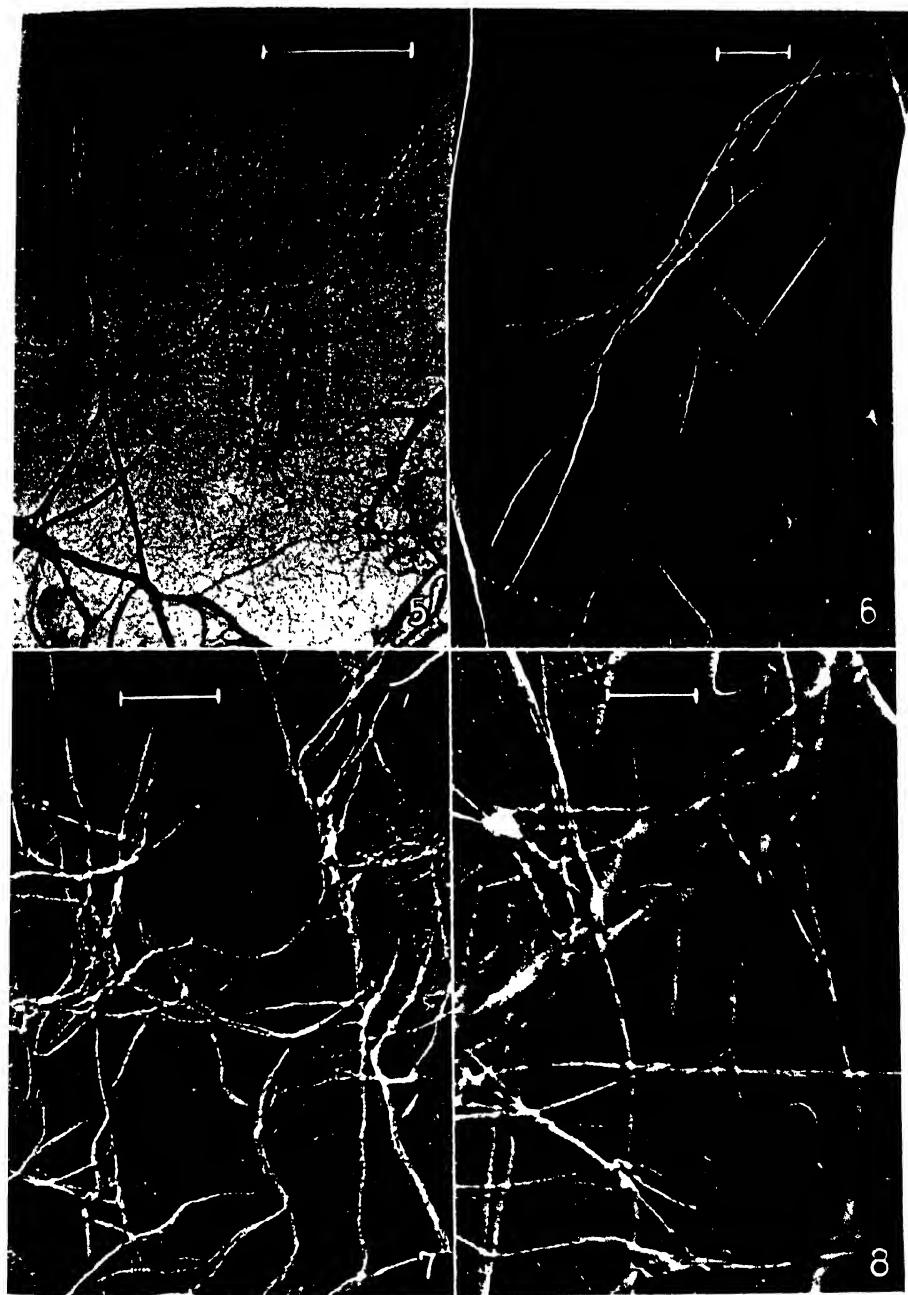
(Porter and Hawn: Formation of clots from fibrinogen and thrombin)

PLATE 23

FIG. 9. This micrograph shows a few strands of a clot formed during 150 seconds. The clotting mixture was the same as that used for the previous preparations. It is presented to show that periodicity of structure developed under the conditions of this study as it had in earlier work. The period measures 250 Å. × 40,500.

FIG. 10. Electron micrograph to show beaded character of dried protofibrils. They were developed in a mixture of fibrinogen and thrombin within 15 seconds after mixing. The width of the fibrils ranges from 200 Å to 230 Å and the average distance between the particles or beads within the fibrils is of the same order. They are shown here for comparison with the model in Fig. 12. × 38,000.

FIG. 11. Electron micrograph of a shadowed preparation of larger fibrin strands to show the beaded or furrowed character of the surface of the mature fiber. The elevations in the fiber surface coincide with the dense striations. Presented for comparison with Fig. 13. × 36,500.

FIG. 12. A model of a protofibril of fibrin, constructed out of disc-shaped unit particles or presumptive molecules. It is proposed that thrombin in some way activates the fibrinogen particles to adhere face to face and polarizes them so that at certain points they attach edge to edge. The shape of the particles is not strictly in keeping with that of the oblate ellipsoid of resolution proposed by Edsall *et al.* (9). For instance, the vertical section is not a smooth ellipse and the vertical axis is too long for the diameter, but for the purposes of this crude interpretation the model is probably adequate. The surface markings on the individual discs are merely incidental to their construction and without significance. × 330,000.

FIG. 13. Model illustrating the proposed development of a small needle-like crystal or unit fiber from the association of three protofibrils such as are shown in Fig. 12. With the disc viewed from the edge in this manner the model accounts for the striated appearance of the unit fiber. The electron image of such a structure would appear striated, however, whether viewed from the edge or face, because the greatest amount of electron-scattering material is concentrated in the central portion of the particle. Presumably the protofibrils can and do associate edgewise as well as face to face. Assuming a periodicity of 250 Å the magnification of the model is about 330,000.
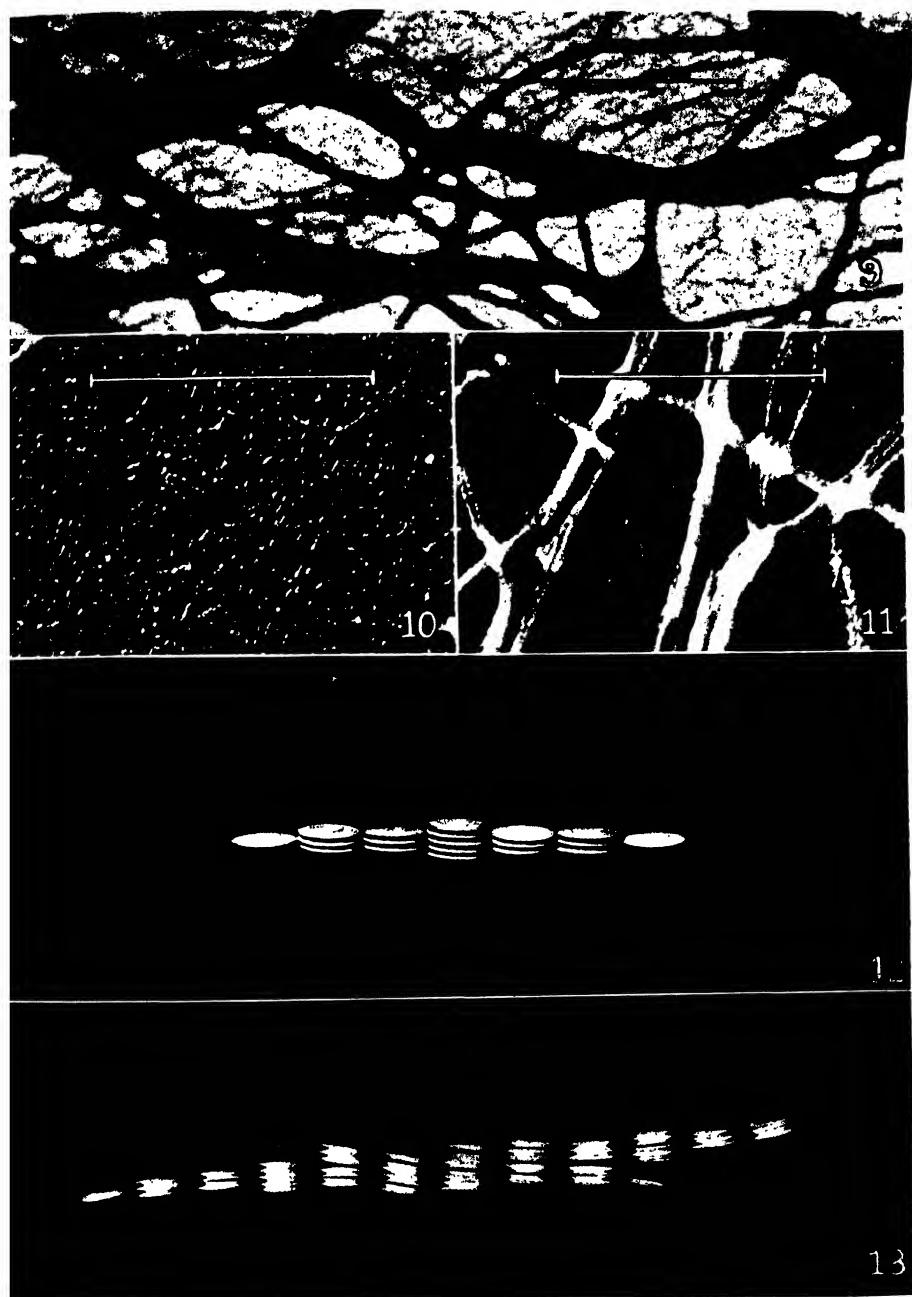
ELECTRON-MICROSCOPY OBSERVATIONS ON HODGKIN'S MATERIAL

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This report is designed to make available to other workers some observations with the electron microscope on cells from Hodgkin's nodes. The study has not been completed but is not being continued at this time.

There are in the literature reports of investigations favoring the virus etiology of Hodgkin's disease (see Hoster and Dratman for a complete review). Since, however, the evidence is by no means conclusive, it seemed of interest to examine representative material by some methods of electron microscopy. We reasoned that, since these methods have demonstrated virus-like bodies in cells suspected of carrying a viral agent,[5, 8] they might be expected to show such, if present, in the cells of Hodgkin's nodes.

Materials and Methods

The biopsies for study were obtained from the Memorial Hospital, New York City. They consisted of the following: fourteen Hodgkin's nodes, one biopsy of a Hodgkin's-involved spleen, one node from a case of Letterer-Siwe's disease, one node containing metastases from a malignant melanoma, and one node from a radical mastectomy case (no metastases present). A number of normal rat nodes were also used.

Table 1 gives, in outline form, the histological characteristics and tissue-culture growth of the human material. All material was cultured on cover-glass inserts in the roller flasks described by Porter, Claude, and Fullam.[9] The nutrient medium contained Tyrode's solution, placental cord serum, and chick-embryo extract. Two media were used, one containing these ingredients in proportions of 1:3:1 and the other in proportions of 5:3:2. The cultures were observed daily and stained preparations were made at various stages of growth. These were stained with hematoxylin and eosin, Giemsa's, and occasionally with Seller's stain. The screens for electron microscopy were prepared by methods now standard for cultured cells.[9]

* On leave of absence from Sloan-Kettering Institute for one year at Rockefeller Institute for Medical Research. A National Cancer Institute Fellow.

TABLE 1

Histological Appearance and Tissue-Culture Growth of Human Material

No.	Material Diagnosis	Histological appearance	Growth in tissue culture
1	Node Hodgkin's	Fibrous note. No eosinophils. Few lymphocytes and Reed-Sternberg cells	Fair outgrowth of macrophages. Few lymphocytes appeared
2	Node Hodgkin's	Very cellular node with many Reed-Sternberg cells and eosinophils; few lymphocytes. A few areas of necrosis	Large outgrowth of macrophages. Few lymphocytes appeared
3	Node Hodgkin's	Very cellular node containing mostly lymphocytes, few Reed-Sternberg cells, and no eosinophils	Very good growth. Many lymphocytes and macrophages present. Large number of giant cells appeared under the explant
4	Node Hodgkin's	Fibrous node containing few Reed-Sternberg cells, rare lymphocytes, and occasional eosinophils	Poor growth. Only a few fibrocytes migrated out of explant
5	Node Hodgkin's	Fibrous node with rare Reed-Sternberg cells and lymphocytes; no eosinophils	No outgrowth from explant
6	Node Hodgkin's*	Fairly cellular node with many Reed-Sternberg cells, few lymphocytes, and occasional eosinophils	Poor growth. Only a few fibrocytes migrated out of the explant
7	Node Hodgkin's	Partly fibrous node with few Reed-Sternberg cells, few lymphocytes, and occasional eosinophils	Poor growth. Only a few fibrocytes appeared
8	Node Hodgkin's	Fairly cellular node containing many lymphocytes, few Reed-Sternberg cells, and no eosinophils	Fair growth. Many lymphocytes and a few macrophages appeared
9	Node Hodgkin's	Very cellular. Many lymphocytes. Few Reed-Sternberg cells. Rare eosinophils	Good growth. Many lymphocytes and macrophages migrated out of explant
10	Spleen Hodgkin's	Very cellular. Large number of Reed-Sternberg cells. Considerable numbers of eosinophils. Few lymphocytes. Many of the reticulum cells contain hemosiderin	Very good growth. Many macrophages present but few lymphocytes. Some of the fibrocytes contain hemosiderin
11	Node Hodgkin's	Very cellular. Many lymphocytes. Few Reed-Sternberg cells present and occasional eosinophils	Fair growth. Few lymphocytes or macrophages appeared

' Patient under treatment with nitrogen mustard.

No.	Material diagnosis	Histological appearance	Growth in tissue culture
12	Node Hodgkin's	Fairly cellular node showing a few small areas of necrosis. Few Reed-Sternberg cells and lymphocytes. Many eosinophils present	Many lymphocytes appeared. Few macrophages. Occasional giant cell present
13	Node Hodgkin's	Fibrous node containing many eosinophils but rare Reed-Sternberg cells and lymphocytes	Fair growth. Very few lymphocytes and macrophages appeared. This culture consisted almost entirely of fibrocytes
14	Node Hodgkin's	Cellular node containing many eosinophils and Reed-Sternberg cells. Lymphocytes present but not in large amount	Culture similar to preceding. Very few lymphocytes and macrophages appeared
15	Node Carcinoma of breast	No metastatic carcinoma cells present	Good growth. Many lymphocytes but few macrophages present
16	Skin nodule Metastatic carcinoma	Very cellular, dense, anaplastic carcinoma	Poor growth. No carcinoma cells grew out—only fibrocytes
17	Node Metastatic melanoma	Few melanoblasts present. Many macrophages containing ingested melanin	Good growth. Few lymphocytes appeared. Many macrophages. A few giant cells formed under explant
18	Node Letterer-Siwe's disease	Very cellular node. Many giant cells present and few lymphocytes. Hyperplasia of reticulo-endothelial elements	Very good growth. Growth much like Hodgkin's. Many macrophages and few lymphocytes appeared. Some giant cells present under explant

Observations on Tissue Cultures and Stained Preparations

All the tissues grew well in tissue culture except four of the Hodgkin's nodes (nos. 4, 5, 6, 7), which gave very poor growth and provided no material for electron microscopy.

On the first two days of culture, numbers of lymphocytes and macrophages migrated out of the explant. The relative amounts of these types varied considerably from node to node. By the second to third day, fibrocytes started to appear around the explant and within a few days became the dominant cell type. Most of the lymphocytes and the few eosinophils that appeared degenerated in a few days, leaving considerable cellular debris that was actively phagocytized by the macrophages and to some extent by the fibrocytes. It is worth noting that this activity was similarly apparent in cultures of non-

Hodgkin's material. The macrophages did not retain their amoeboid existence very long, but soon attached themselves to the cover-slip and assumed a fibrocyte-like form. Giant cells ultimately appeared in four cultures: two of Hodgkin's nodes, one the node containing metastatic melanoma, and the other the Letterer-Siwe's node (nos. 3, 12, 17, 18). These were found under the main explant and had all the morphological characteristics of foreign-body giant cells. They did not resemble the Reed-Sternberg cell. In one case, a giant cell was found containing as many as fifty nuclei. The nuclei were all uniform in size, no larger than the macrophage nuclei, and appeared normal.

The Reed-Sternberg cell (the multinucleated type or the one with a multilobulated nucleus) did not appear to migrate out of the explant. In none of the cultures could we identify any cell that we could definitely say was a Reed-Sternberg cell, although sections of the biopsies showed these cells were present in the Hodgkin's material. This is, of course, important in the interpretation of our results.

In some of the Hodgkin's cultures, we occasionally found fibrocytes with nuclei up to twice the normal size. However, these nuclei were oval and regular in shape in contrast to the misshapen nuclei of the Reed-Sternberg cell. It is possible that they represent those cells in the Hodgkin's nodes with somewhat large, vesicular nuclei that are not yet lobulated or indented. We did not encounter any striking examples of these cells on the electron-microscope screens, because they were so few in number.

From one of the cultures, we obtained definite evidence concerning the histological origin of the fibrocytes in spleen and lymph-node cultures; sections of the Hodgkin's nodules from the splenic biopsy showed that many of the reticulum cells contained hemosiderin. Some of the fibrocytes that grew out of these nodules contained hemosiderin and these were morphologically indistinguishable from the nonhemosiderin-containing fibrocytes. Since the reticulum cells of the lymph nodes and spleen are indistinguishable, it is probable that the fibrocyte growing out of each of these tissues has the same origin, namely, the reticulum cell.

Electron-Microscope Observations

Electron-microscope screens were prepared from all cultures that grew well, and all cells on the screens were examined at a magnification of 2,600 to 4,200. The cell types present on the screens consisted of the fibrocytes and foreign-body giant cells described in the previous section. Electron micrographs were taken of parts of many of the cells and enlarged to a magnification of 7,000 to 17,000 for further study. There were no constant differences in cytomorphology between the cells from the Hodgkin's tissue and the other tissues, normal and neoplastic. In none of the cells from the Hodgkin's or the other material was any particulate body found with the characteristics of a virus (see[5, 8]).

CONCLUSION

Our data, incomplete as they are, favor the nonviral etiology of Hodgkin's disease, but our observations are limited. Most pathologists consider the Reed-Sternberg cell an essential criterion for the definite diagnosis of Hodgkin's disease. Our inability to obtain the Reed-Sternberg cell for electron-microscope study thus leaves the possibility that the hypothetical virus is confined to this cell. High cell specificity is usually associated with viruses and has been demonstrated in tissue culture. Carrel[1, 4] showed that in pure chicken-fibrocyte cultures inoculated with the agent of Chicken Tumor I, the virus rapidly disappeared from the culture fluid, whereas in leukocyte cultures, this same agent multiplied readily. In further work,[2, 3] he demonstrated that strains of fibrocytes obtained from Chicken Tumor I rarely produced tumors when inoculated into chickens, whereas strains of macrophages from the same tumors almost always induced tumors. Similarly, Hallauer showed that the fowl-plague virus multiplies in cultures containing chick-embryo skin, iris epithelium, and brain but not in pure cultures of fibrocytes or blood monocytes.

Some method is needed by which to obtain sufficient numbers of the Reed-Sternberg cells in tissue culture with the cells well spread so they are not too dense to the electron beam. This, coupled with a sufficiently large sample of Hodgkin's nodes of good quality for culturing, should make possible the resolution of the problem.

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ROLE OF CO-FACTORS IN THE INHIBITION OF BACTERIAL VIRUSES BY THE SOMATIC ANTIGENS OF SHIGELLA SONNEI

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During the course of an investigation[1] on the susceptibility of *Sh. sonnei* Phase I and II[2] and their phage-resistant variants to the T series of bacterial viruses, it was found that the Phase I micro-organism was lysed only by T_2 and T_6, whereas the Phase II bacillus was susceptible to T_3, T_4 and T_7 as well as T_2 and T_6. Solutions of the chemically purified and immunologically specific somatic antigens derived from Phase I and II bacilli[3] were incubated at 37° C. with approximately 2,000 particles per ml. of the appropriate viruses in an ammonium lactate medium (F)[4]. The bacteriophages employed were kindly supplied by Dr. Mark Adams, of New York University. Fresh stocks of the T_3 and T_7 viruses were prepared in nutrient broth using *E. coli* B as the host cell. T_2 and T_6 were prepared in F medium, and the T_4 virus in F medium containing 5 micrograms of 1-tryptophane per ml. When 0.1 ml. of the mixture was assayed on nutrient agar by the Hershey poured-plate technique[5], there was no evidence that the lipocarbohydrate–protein antigens, even in concentrations as high as 0.1 mgm. per ml., would inhibit the lytic action of the viruses. This indicated that the purified antigens were incapable of preventing adsorption of the virus by the bacterial cell. These observations appear to be contrary to those of other investigators[6], who found that extracts of a variety of micro-organisms, which presumably contained immunologically active carbohydrates, have the power of preventing the adsorption of bacterial viruses to the host cell.

Further investigation has now revealed that the somatic antigen of Phase II *Sh. sonnei* in the presence of broth does inhibit the lytic action of T_3, T_4 and T_7 on the homologous micro-organism. On the other hand, the same antigen fails to inhibit the action of T_2 and T_6. It appeared that some constituent of the broth plays an essential part in the selective inhibition of T_3, T_4 and T_7 by the Phase II antigen. Anderson[7] has shown that 1-tryptophane serves as a co-factor in promoting the absorption of T_4 and T_6 on *E. coli*. It seemed possible that this amino-acid might likewise be involved in the combination of T_3, T_4 and T_7 with the cell-free somatic antigen derived from *Sh. sonnei* Phase II.

Inhibition of Bacterial Viruses T_3, T_4 and T_7 by the Somatic Antigen of Phase II Sh. sonnei

Bacterial virus tested	Medium used	Final concentration of Phase II antigen in virus-antigen mixture*		
		0.1 mgm.	0.01 mgm.	0.001 mgm.
T_3	F	0	0	0
	Broth	99	65	12
	F plus 0.2 mgm. 1-tryptophane per ml.	0	0	0
T_4	F	0	0	0
	Broth	99	96	93
	F plus 0.2 mgm. 1-tryptophane per ml.	98	85	63
T_7	F	0	0	0
	Broth	98	93	81
	F plus 0.2 mgm. 1-tryptophane per ml.	0	0	0

* The figures represent the percentage of virus inactivated. F = ammonium lactate medium.

When 0.2 mgm. of 1-tryptophane per ml. was added to F, as little as 1 microgram per ml. of the Phase II antigen inhibited T_4, but had no effect upon T_3 and T_7, as can be seen from the accompanying table. Since the latter viruses are inhibited by comparable quantities of the Phase II antigen in the presence of broth, it would appear that in these instances still other co-factors are involved.

The antigen of Phase I *Sh. sonnei* caused no inhibition of T_2 and T_6 when tested with Phase I micro-organisms, or of T_3, T_4 and T_7 when tested with Phase II bacilli, even in the presence of broth. It is evident, therefore, that the inhibitory action of the Phase II antigen is highly specific, and that a co-factor is required in order for it to block combination of T_3, T_4 and T_7 with the host cell.

These experiments leave certain facts still unexplained. It is not yet known whether the virus combines with the protein, the lipid or the carbohydrate constituent of the somatic antigen; but work now in progress should establish this point. The nature of the co-factors present in broth which permits T_3 and T_7 to be inhibited by the antigen is not yet known. It would appear, however, that in the case of Phase II *Sh. sonnei* the substance which combines with T_3, T_4 or T_7 is a constituent of the lipocarbohydrate–protein complex distributed at the bacterial surface, and that this combination occurs only when an essential co-factor is present.

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STUDIES ON BACTERIOPHAGE

I. The Relationship between the Somatic Antigens of Shigella sonnei and Their Susceptibility to Bacterial Viruses*

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The chemical nature of the receptor groups on the surface of microorganisms susceptible to lysis by bacteriophages has never been fully established. The hypothesis that the sensitivity of certain bacteria to phages can be correlated with their thermostable agglutinogens is attractive, and a number of investigators have presented evidence indicative of such a relationship. The fact remains, however, that with but a single exception (1), no one has obtained from bacterial cells a well defined chemical fraction the nature of which has been unequivocally established and which has the capacity to combine with a bacterial virus and block its action on the host cell.

It was Hadley (2) who first pointed out that three serologically related microorganisms, *B. pullorum, B. gallinarum, B. typhosus*, are all sensitive to the same bacteriophage. This observation was confirmed and extended by Burnet (3) who concluded that Salmonella types possessing the same heat-stable agglutinogen as exists in *B. enteriditis* reacted similarly to a series of phages. Transition of the microorganism from S to R was accompanied by changes in phage susceptibility, which in turn were correlated with the presence or absence of the O antigen. In addition, it was pointed out that R variants of different Salmonella types, all sharing a common R antigen, were lysed by a phage developed on any one of the R strains. Similarly the phage sensitivity of smooth variants of Flexner dysentery bacilli appeared to be related to the presence of the type-specific somatic antigen, and those strains which were antigenically similar showed reactions toward a series of bacteriophages which were nearly identical (4). Because of these relationships, it was suggested that the constituent on the surface of the bacterial cell which specifically combines with phage is identical with the heat-stable agglutinogen and it was implied that the carbohydrate hapten component served as the virus receptor site. Certain discrepancies, however, made it difficult to accept this hypothesis for it was possible to obtain phage-resistant variants which showed no serological change from the parent cell, and in addition rough variants were often observed to show variations in their phage patterns. These objections were ingeniously met by the suggestion that slight changes in antigenic structure could come about which are not detectable by the usual serological tech-

niques (5), but only by sensitivity of the cell to phage, a concept not wholly compatible with our concepts of immunological specificity. By employing artificially compounded antigens containing saccharides of known chemical constitution it has been shown in this laboratory that even the subtlest of differences in the structure of carbohydrates can be readily detected by appropriate serological means (6).

Levine and Frisch (7) were the first to show that extracts of certain microorganisms had the ability to inhibit the lytic action of bacteriophages. On the basis of incomplete chemical evidence they concluded that the active principle present in these extracts was carbohydrate in nature. Attempts to purify phage inhibitors prepared by extracting microorganisms in one way or another have on the whole been unsuccessful (1). In fact, one well characterized bacterial product, the somatic antigen of the Shiga bacillus prepared by Morgan (8), was found to be devoid of specific phage-inhibiting activity (9).

In résumé one can conclude on the basis of excellent serological evidence that the phage susceptibility of certain Gram-negative microorganisms can be intimately correlated with their thermostable antigens, but the chemical work which has been presented gives no ground for the assumption that these substances function as the virus receptor sites of susceptible host cells.

In recent years knowledge concerning the chemical nature of bacterial polysaccharides and of the somatic antigens of the enteric group of microorganisms has been greatly advanced (10). In addition, knowledge of bacterial viruses, their classification, morphology, and host relationships has likewise been greatly broadened. In particular, the so called T series of bacteriophages and their relationship to the B strain of *E. coli* have been extensively studied (11).

Recently a study of two strains of *Sh. sonnei*, Phase I and Phase II, has been made (12). These microorganisms are type-specific and show little or no serological crossing despite the fact that the Phase II bacillus arises from the Phase I organism by spontaneous mutation. A detailed chemical investigation of the somatic antigens of these two microorganisms has revealed that they are chemically similar yet immunologically distinct and specific. The purified antigens have been isolated in an electrophoretically pure form and have been characterized as lipocarbohydrate-protein complexes. It is these substances which confer upon the parent cell the property of type specificity. Whether they are concerned with the susceptibility of *Sh. sonnei* to the T series of bacteriophages is not known, but this experimental material appears to provide an excellent opportunity to learn something of virus-host relationships.

A study was undertaken therefore to determine first, whether differences in the phage resistance patterns of Phase I and II *Sh. sonnei* could be established, and if so, whether such differences had any relationship to the somatic antigens of these two microorganisms. The results of these investigations are presented in the following account.

EXPERIMENTAL
Methods and Materials

Strains of Microorganisms.—The two type-specific strains of *Sh. sonnei* (Phase I and II$_s$) used were obtained from the United States Army Medical School. They had the morphological and serological characteristics previously described (12). The Phase II$_s$ *Sh. sonnei*, for sake of brevity, will be referred to in this communication as Phase II. The strain B of *E. coli* which has been used by many investigators for much of the work with the T series of bacteriophages was secured from Dr. Mark Adams of the Department of Microbiology of New York University.

Media.—The media employed were:—

(*a*) Nutrient broth prepared by dissolving 8 gm. of desiccated Difco nutrient broth and 5 gm. of sodium chloride in a liter of distilled water.

(*b*) 1.5 and 0.8 per cent nutrient agar were obtained by adding the appropriate amount of Bacto agar to the above medium.

(*c*) *F medium* prepared as described by Schlesinger (13).

(*d*) *S medium.*—This is a synthetic medium which maintains the growth of dysentery bacilli. It was prepared essentially according to the directions of Dorfman and Koser (14), as follows:—

$(NH_4)_2HPO_4$...	2.0 gm.
KH_2PO_4...	1.5 ”
NaCl...	5.0 ”
$MgSO_4 \cdot 7H_2O$.......................................	0.1 ”
Distilled water...	1 liter

This solution was autoclaved and to it was added 10.0 ml. of a solution containing 2.5 gm. of glucose and 0.05 mg. of nicotinic acid sterilized by filtration.

Bacteriophages.—The bacteriophages employed were the well known T series and were kindly supplied by Dr. Mark Adams. Stocks of T$_3$ and T$_7$ were prepared in broth using *E. coli* B as the host cell. T$_1$, T$_2$, T$_5$, and T$_6$ were prepared in a similar manner in "F" medium. Two separate stocks of T$_4$ virus were prepared. The first, a tryptophane-requiring variant, was obtained essentially by the technique of Delbrück (15) and has been designated as T$_4$ tr. A second stock, designated as T$_4$ and which did not require tryptophane as a cofactor was prepared as follows:—

A non-tryptophane-requiring T$_4$ virus obtained from Dr. Adams and produced on *E. coli* in "F" medium, was plated on S medium using Phase II *Sh. sonnei* as host. A single plaque was picked and used to infect a culture of the Phase II organism in S medium. The lysate was filtered and assayed. The method of bacteriophage assay used throughout the experimental work was that described by Hershey (16).

Somatic Antigens of Phase I and II Sh. sonnei.—Highly purified preparations of these two substances (12) were kindly supplied by Dr. Edgar E. Baker of the Rockefeller Institute.

Susceptibility of Phase I and II Sh. sonnei to the T Series of Bacteriophages

It has been suggested that the receptor for the adsorption of bacterial viruses to the surface of certain microorganisms is identical with the thermostable agglutinogen (3, 4). If this is true then the Phase I and II *Sh. sonnei*, which elaborate different type-specific somatic antigens, should differ in their susceptibility to the T series of bacteriophages. The sensitivity of these microorganisms was therefore determined as follows:—

A high concentration of phage, 0.05 ml. of stock containing about 10^9 particles per ml., was spread on the surface of a nutrient agar plate in the presence of the test organism, incubated overnight at 37°C., and examined the following morning. If the organism was sensitive there was complete lysis except for a few resistant mutants. If the organism was resistant it showed no lysis. The results are recorded in Table I.

It can be seen that the Phase I organism was lysed only by the T_2 and T_6 viruses and was resistant to the remaining phages. The Phase II bacillus, on the other hand, was lysed by all the phages save T_1 and T_5. Thus, it is apparent that Phase I and II *Sh. sonnei* are not identical in their sensitivity to the bacteriophages. It will be shown that these differences can in some, but not all, instances be correlated with the type-specific somatic antigens.

TABLE I

Sensitivity of Phase I and II Sh. sonnei to the T Series of Bacteriophage

Phage type	Bacterial culture	
	Phase I	Phase II
T_1	R	R
T_2	LM	L
T_3	R	L M
T_4	R	L M
T_5	R	R
T_6	L M*	L M
T_7	R	L M

L = lysis by the phage. R = resistant to the phage. M = mutants present.
* = a great many mutants were produced.

Inhibition of the Adsorption of Bacteriophage by the Somatic Antigens of Phase I and II Sh. sonnei

If the somatic antigen is the receptor for bacterial viruses it should be possible to inhibit or block adsorption of phage on the susceptible host cell with solutions of these type-specific lipocarbohydrate-protein complexes in the following manner:—

A sterile solution of antigen containing 2 mg. per ml. was prepared by wetting a weighed sample with 0.5 ml. of 70 per cent alcohol. After a few minutes the appropriate quantity of sterile 0.1 M phosphate buffer at pH 7.0 was added. Dilutions of the antigen stock were made in the particular medium selected. The virus to be tested was diluted in the same medium so that 1 ml. contained roughly 4×10^3 particles. Mixtures of 1 ml. of virus dilution and 1 ml. of each antigen dilution were made and incubated at 37°C. overnight. 0.1 ml. of mixture was then plated on nutrient agar after the addition of 0.3 ml. of a suspension of the bacterial cell under investigation. The plates were incubated at 37°C. overnight and plaque counts were made the following morning. The inhibitory action of the two somatic antigens was tested on all the phages to which the Phase I and II *Sh. sonnei* were susceptible. The experimental results are recorded in Table II.

It can be seen that lysis of the Phase I organism by the T_2 and T_6 viruses was not inhibited by the homologous somatic antigen, nor was lysis of the Phase II organism by these viruses specifically inhibited by the Phase II so-

TABLE II

Inactivation of Bacteriophages by Somatic Antigens of Phase I and II Sh. sonnei

Sh. sonnei	Antigen	Diluent used	Bacterio-phages tested	Final concentration of antigen in virus-antigen mixture*			
				1 mg.	0.1 mg.	0.01 mg.	0.001 mg.
Phase I	Phase I	Nutrient broth	T_2	0	0	0	0
			T_6	0	0	0	0
"	"	F‡	T_2	0	0	0	0
			T_6	0	0	0	0
Phase II	Phase II	Nutrient broth	T_2	0	0	0	0
			T_3	100	99	65	11
			T_4tr	97	99	99	91
			T_6	0	0	0	0
			T^7	99	98	94	79
"	"	F	T_2	0	0	0	0
			T_3	95	0	0	0
			T_4	100	98	85	63
			T_4tr	33	0	0	0
			T_6	0	0	0	0
			T_7	56	0	0	0
"	"	F + 0.2 mg. l-tryptophane	T_4	100	100	100	83
			T_4tr	97	98	94	22
"	"	S§ S + 0.2 mg. l-tryptophane	T_4	99	99	96	69
			T_4tr	99	99	83	7

* In this and in subsequent tables the figures represent per cent of virus inactivated.
‡ Dilutions were made in F medium, platings were made on nutrient agar.
§ Dilutions were made in S medium, plating on S agar.

matic antigen. In contrast the T_3, T_4 tr, and T_7 viruses, in the presence of nutrient broth, were inhibited by the Phase II somatic antigen, for when the susceptible host cell was added to the virus-antigen mixtures and then plated, there was no formation of plaques. The latter viruses were in each instance bound by the Phase II antigen; their activity was blocked by concentrations of the lipocarbohydrate-protein as low as 10 micrograms per ml. and even with

concentrations as low as 1 microgram per ml. there was considerable viral inhibition. The latter did not take place, however, if virus and antigen were incubated in a synthetic medium. Combination apparently occurred only in the presence of appropriate cofactors supplied by the nutrient broth. One exception, however, was the T_4 virus, a non-tryptophane–requiring stock which was inactivated, as one might expect, in a synthetic medium devoid of tryptophane. Inhibition of lysis of Phase II *Sh. sonnei* by the T_3, T_4 tr, and T_7 viruses was specific and was affected only by the homologous Phase II antigen and not by the Phase I antigen, as is evident from the results presented in Table III. The significance of these observations will be discussed below.

TABLE III

Specificity of Inactivation of T_3, T_4, and T_7 Bacteriophages by the Somatic Antigens of Phase I and II Sh. sonnei

Host cell	Antigen used	Bacteriophage tested	Final concentration of antigen in virus-antigen mixture			
			1 mg.	0.1 mg.	0.01 mg.	0.001 mg.
Sh. sonnei Phase II	Phase II	T_3	100	99	65	11
		T_4tr*	97	99	97	88
		T_7	99	98	94	79
,, ,,	Phase I	T_3	0	0	0	0
		T_4tr	0	0	0	0
		T_7	0	0	0	0

* T_4tr—This stock of virus was prepared in F medium containing *l*-tryptophane and using *E. coli* B as host cell.

Rate of Inhibition of T_3, T_4, and T_7 by the Somatic Antigen of Phase II Sh. sonnei

The fact that three of the viruses, T_3, T_4, and T_7, were inhibited by the somatic antigen of Phase II *Sh. sonnei* would indicate that chemical combination between virus and antigen had taken place. It seemed of interest therefore to determine the rate of formation of the virus-antigen complex as measured by viral inhibition.

To a standard dilution in S medium of the non-tryptophane–requiring phage T_4 containing approximately 5×10^3 virus particles per ml. was added an equal volume of a solution of the Phase II somatic antigen, in S medium, containing 200 micrograms per ml. The mixture was maintained at 37°C. 0.1 ml. was removed at varying time intervals and assayed by the poured plate technique, using the Phase II organism as host cell. The plaques were counted following 18 hours incubation at 37°C.

It is apparent from the results presented in Table IV that at the high concentration of inhibitor used (100 micrograms per ml.) combination between virus and antigen occurred very rapidly. Some 94 per cent of the virus was

inhibited at zero time as can be seen in Table IV. There was no marked increase of inactivation following further incubation of virus and antigen. The rate of inactivation of T_3 and T_7 was also studied, but in these instances it was necessary to use broth as the diluent, since both viruses require a cofactor. It can be seen that in both cases inactivation was slower than with the T_4 virus. Whether this difference in rate is due to the fact that in the latter instance it was possible to carry out the reaction in a synthetic medium, is not known. In order to determine with precision the velocity constant of the inactivation of the three phages by the somatic antigen, it would of course be necessary to use far smaller quantities of the latter.

Activity of Phase II Somatic Antigen after Enzymatic Degradation

The ability of the Phase II antigen to inhibit the various T phages may be an attribute of the intact complex, or attributable to one of its constituents.

TABLE IV

Rate of Inactivation of T_3, T_4, and T_7 Bacteriophages by the Somatic Antigen of Phase II Sh. sonnei

Bacteriophage	Hours incubation of phage inhibitor mixture prior to plating					
	0	0.5	1	2	4	6
T_3	—	—	73	82	80	87
T_4	94	97	97	98	99	—
T_7	—	—	80	93	99	99

T_3 and T_7 were diluted in nutrient broth and plated on nutrient agar.

T_4 was diluted in S medium and plated on S agar.

Chemical degradation of the complex into its protein, lipid, and carbohydrate components can be effected only by fairly drastic procedures. After it was observed that the mere heating of a solution of the antigen at 100°C. in 0.1 M phosphate buffer at pH 7.0 for 40 minutes sufficed to destroy some 90 per cent of its phage-inhibiting activity, it was thought that heating at 100°C. for 4 hours in 1 per cent acetic acid, the standard procedure for complete dissociation of the complex, could yield only degraded and inactive end products. This proved indeed to be the case. It was possible, however, to degrade the lipocarbohydrate-protein complex by a less drastic procedure; namely, through enzymatic degradation of the protein component by means of proteolytic enzymes. For this purpose pancreatin was selected because it contains a variety of proteolytic enzymes including polypeptidases.

0.15 gm. of the Phase II somatic antigen was dissolved in 15 ml. of 0.1 M phosphate buffer at pH 7.8 and 10 mg. of Parke Davis pancreatin was added. The solution was placed in a cellophane bag together with 1 ml. of toluene, and dialyzed at 37°C. for 3 days against suc-

cessive changes of the same buffer. After adjusting the pH to 5.0 with 1 N acetic acid the solution was deproteinized by shaking five times with a chloroform-octyl alcohol mixture. After thorough dialysis against successive changes of distilled water, the deproteinized solution was concentrated by pervaporation, and the material isolated by drying from the frozen state, 0.1 gm. was recovered.

This material, which gave none of the common protein reactions, still contained the native carbohydrate hapten bound to phospholipid. A 1 per cent solution of this substance gave neither a ninhydrin nor a biuret test and failed to show absorption typical of the protein component of the parent antigen when examined spectroscopically in the ultraviolet. A quantitative photometric determination (17) of its specific serological activity revealed that the substance had an activity, on the basis of dry weight, somewhat greater than that of the undegraded antigen. When tested for its ability to inhibit the activity of the T_4 phage on Phase II *Sh. sonnei*, it was found that this substance was still capable of inhibiting the bacteriophage. A quantitative comparison of the phage-inhibiting ability of the native and of the enzymatically degraded antigen showed no striking differences. Thus, it would appear that the component of the Phase II somatic antigen responsible for inhibiting the action of the phages in question is probably the carbohydrate hapten.

<div align="center">DISCUSSION</div>

The experimental evidence obtained during the course of this investigation lends support to the concept that there is a relationship between the phage sensitivity of smooth variants of Gram-negative bacilli and their somatic antigens. The fact that we have at hand the type-specific antigens of two microorganisms of the same species, and that well characterized bacterial viruses are likewise available, has made it possible to put to test both the validity of this hypothesis, and within certain limits, its scope as well.

It will be recalled that *Sh. sonnei* Phase II was lysed not only by the T_2 and T_6 bacteriophages, but by the T_3, T_4, and T_7 viruses as well. The Phase I bacillus, on the other hand, was sensitive only to T_2 and T_6. It was shown, furthermore, that lysis of Phase II bacilli by the T_3, T_4, and T_7 viruses could be readily inhibited by the homologous Phase II somatic antigen, but not by the heterologous. In neither instance, however, could lysis of the Phase I and II microorganisms by the T_2 and T_6 viruses be inhibited by either of the homologous bacterial antigens. This sharp demarcation in behavior of the T_3, T_4, and T_7 viruses as opposed to the T_2 and T_6 phages suggests that the receptor substance for the two groups of viruses is different.

In the case of Phase II *Sh. sonnei* it would appear, from the evidence presented, that the receptor substance at the surface of the bacterial cell with which the T_3, T_4, T_4 tr, and T_7 viruses combine prior to lysis is the type-specific lipocarbohydrate-protein complex. It is not this substance, however,

which serves a similar function for the T_2 and T_6 viruses, for their lytic action on the Phase II bacillus is not inhibited by the somatic antigen. Nor is their ability to combine with and cause lysis of Phase I bacilli inhibited by the Phase I antigen. It becomes necessary therefore to assume that the T_2 and T_6 viruses combine with Phase I and Phase II *Sh. sonnei* by virtue of substances other than their respective lipocarbohydrate-protein antigens. The chemical nature of this receptor is as yet obscure. In this respect the two groups of bacterial viruses, T_2 and T_6 on the one hand, the T_3, T_4, and T_7 on the other, exhibit differences in tropism analogous to those shown by certain animal viruses. Such a concept entails the assumption that the cell surfaces of bacteria possess mosaics of well defined chemical components each exhibiting specific affinities for the virus in question. When mutation occurs the genetic constitution of the bacterial cell is altered so that the factors involved in the synthesis of one or more of the antigens comprising the cell surface mosaic are also changed. It may well be that these changes can in some instances be so subtle as to be detected only by phage susceptibility as suggested by Burnet. It appears to us, however, that a thorough study of antigens actually isolated from such mutants should reveal not only differences in their chemical structure which can be correlated with changes in phage susceptibility, but that differences in the immunological specificities of the antigens of mutants and parent cells may likewise reveal themselves.

In a communication presented by Maurer and Woolley (18) it was shown that the addition of citrus pectin to a medium which supported the growth of *E. coli* protected the microorganism against the bacteriophage T_2. It was pointed out, furthermore, that the same phenomenon could be observed when gelatin or an enzymatic degradation product of this substance was employed. The amount of citrus pectin necessary to protect the bacterial cells was large, the lowest effective concentration being 10 mg. per ml. An analysis of these observations revealed, however, that the bacterial virus was neither inhibited by the citrus polysaccharide to the extent that it failed to be absorbed by the host cell, nor did it even inhibit the multiplication of the virus. The carbohydrate did, however, inhibit lysis of the infected cells and allowed them to grow and multiply in the presence of the phage. In this connection it is of interest that Price (19), working with *Staphylococcus muscae* and another strain of phage, has shown that in a synthetic medium containing casein hydrolysate the release of virus following a very low multiple infection is not correlated with observable cellular lysis.

It is difficult to conceive of the phenomenon described above as being comparable to that presented in this communication. In the instances described here, the inactivation of a given virus in respect to its host cell is accomplished by exceedingly minute amounts of polysaccharide or of a polysaccharide-containing antigen, one microgram or less, and it must have the appropriate

chemical structure. Not only is this inhibition phenomenon highly specific, but it is dependent upon the nature of the receptor as well.

In conclusion, it should be emphasized that when the inhibition experiments described in this communication were performed in synthetic media, they were unsuccessful except in the case of the non-tryptophane–requiring T_4 virus. With the other viruses employed it was necessary to have essential cofactors present in order that the virus-antigen complex could be consummated (20).

SUMMARY

Phase I and Phase II *Sh. sonnei* exhibit differences in their susceptibility to the T series of bacteriophages. Both microorganisms are lysed by T_2 and T_6, but only the Phase II bacillus is lysed by T_3, T_4, and T_7.

Lysis of the Phase I or Phase II bacillus by T_2 or T_6 is not inhibited by the homologous type-specific antigen. In the presence of an appropriate cofactor, however, the lysis of Phase II *Sh. sonnei* by T_3, T_4, and T_7 is specifically inhibited by the homologous somatic antigen but not by the Phase I antigen.

The significance of these observations is discussed in respect to the nature of the virus receptor of these microorganisms.

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THE NATURE OF THE GOLGI APPARATUS

I. Parallelism between Intracellular Myelin Figures and Golgi Apparatus in Somatic Cells*

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TWENTY-SIX FIGURES

In 1898 Camillio Golgi demonstrated in the pericarya of nerve cells a new intracellular structure referred to by him as the internal reticular apparatus (Golgi, 1898a and b; 1899). In the course of the subsequent 50 years, more than 2,000 papers have been devoted to the description and interpretation of this structure (Hibbard, '45), and resounding controversies, concerning its origin and significance, arose among cytologists. Under the prevailing name of "Golgi apparatus," it has been accepted as a constant organelle of normal animal cells. Supposedly homologous structures have been described in plant cells (Bowen, '28).

Following the original Golgi procedure, which consisted in fixation with an osmium tetroxide-potassium dichromate mixture and subsequent silver impregnation, a variety of methods, still based on silver impregnation, have been proposed for the proper demonstration of the apparatus (Cajal, '12; da Fano, '20; Aoyama, '29). A number of techniques, using the OsO_4 as principal reagent, were introduced after it had been found that slow blackening of the organelle took place in tissues left for prolonged periods in "osmic acid" solutions (Kopsch, '02).

The morphology of the apparatus is characterized by a remarkable polymorphism and a wide variability. An important physiological role has been ascribed to this organelle which has been connected with secretory processes (Nassonov, '23, '24; Bowen, '26, '29), its continuously changing appearances being supposed to represent definite phases in a secretory cycle.

Efforts to demonstrate a Golgi apparatus in living, or fresh, somatic cells have been unsuccessful, except for a few and doubtful cases concerning negative images encountered in tissue culture material (Champy, '26; Champy and Morita, '28; Ludford, '35). Some of these images were later recognized as present only in degenerating or vitally-stained elements (Ludford, '42).

The situation is significantly different in germ cells. In spermatocytes and spermatids there is a conspicuous structure composed of a discontinuous shell of refringent rods or scales (dictyosomes), enveloping a mass of less refringent

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material (idiosome, or archoplasm). This complex structure has been identified with a typical Golgi apparatus because of the osmiophily of its dictyosomes and a secretory activity presumably exerted in relation with the production of the acrosome. Such a formation, however, differs in important respects from the usual Golgi apparatus (Hibbard, '45); unlike the latter, the dictyosomes exhibit, both vitally and after fixation, the same staining affinities as mitochondria; they are stable structures, lacking the well-known Golgi polymorphism; and, above all, they are readily observed in living cells. The evidence, therefore, does not justify their inclusion among the usual Golgi apparatus. It is probable that these structures represent differentiated organelles peculiar to this specialized cell line. In living oocytes and ova, the apparatus is represented by a large number of scattered granules or vesicles which maintain generally their appearance after fixation and impregnation.

At variance with the situation in germ cells, fresh somatic cells do not show anything similar to the impregnated images of the apparatus. A large discrepancy exists between the simple accumulations of refringent droplets found in living or fresh condition and the complicated structures which appear in their place following appropriate treatment. This discrepancy has led a number of cytologists to question the reality of the organelle and to consider the Golgi apparatus as an artifact, resulting from the coalescence (Parat, '28), or the deformation (Worley, '46) of cytoplasmic vacuoles, stainable vitally with certain basic dyes.

Together, the preceding observations and considerations strongly support the hypothesis that, in somatic cells, the Golgi apparatus is an artifact induced by the histological procedures considered as necessary and appropriate for its demonstration.

The purpose of the present paper is to demonstrate that the refringent droplets found in living somatic cells, precisely in their so-called "Golgi zones," can transform into characteristic polymorphic structures, under the influence of favorable concentrations of ethanol. The structures so produced have been identified as myelin figures. In the cells examined; these intracellular myelin figures have been found to duplicate closely, in morphology and topography, the various Golgi apparatus appearances described in the respective cell types by the cytological literature.

The following presentation of the results will include: (1) a description of the lipid inclusions, as they appear in living, or fresh vertebrate somatic cells; (2) a description of the progressive changes induced in the morphology of the same lipid inclusions under the influence of ethanol.

Materials and Methods

Materials

The following organs and tissues have been used in these studies: (a) *mammalian:* rat liver, pancreas, intestinal epithelium, kidney, epididymis, brain, and spinal

ganglia; rabbit cord and spinal ganglia; (b) *avian:* gizzard epithelium and muscularis of newly hatched and adult chicken, and chick embryo brain (17 days old).

The mammals were killed through a blow on the head, the chick embryos through bleeding. The organs were collected and homogenized immediately in different media. The major part of the work has been carried out with liver homogenates.

Methods

Preparation of homogenates. The organs were forced through a masher fitted with a 1 mm mesh screen, and the resulting pulp was suspended in one of the following solutions: (a) 0.07 M NaCl, (b) 0.15 M (isotonic) NaCl, (c) 0.25 M (isotonic) sucrose, or (d) 0.88 M sucrose. The proportions were approximately 4 cm^3 of ice-cold solution per gram of tissue pulp. The suspensions were homogenized, at room temperature, in an "all-glass apparatus" (Potter and Elvehjem, '36) turning at approximately 1,000 rotations per minute. In order to preserve a large number of intact cells, a pestle was chosen which fitted but loosely the tube of the homogenizer. The time required for suitable homogenization varied from 30 seconds to two minutes, depending on the type of tissue. It was determined by repeated microscopical examination, or else, by the aspect of the suspension which should appear homogeneously opaque, without coarse tissue fragments. The preparations were kept thereafter at ice-box temperature (about 4°C.). A homogenate prepared in this manner contains in suspension small clumps of cells, isolated cells, cell fragments, and free cell constituents such as nuclei, mitochondria, zymogen granules and lipid droplets.

No special difficulties were encountered in the preparation of most tissue homogenates. For some organs, containing large amounts of fibrous and muscular tisssue, as the epididymis, preliminary mincing was necessary before forcing through the masher. For gizzard mucosa preparations, it was found preferable to use newly hatched chicken, in which the mucosa could be more readily detached from the subjacent muscularis, than in adults. The mucosa layer was stripped of its superficial keratin coating, finely minced, and then dispersed directly in the homogenizer without the usual step through the masher which, in this case, was found unnecessary. Rat intestine was cut open and the exposed mucosa washed repeatedly with saline, or sucrose solution, in order to remove most of the mucus, which would otherwise interfere subsequently with a uniform contact between the cellular material and the various reagents tested. The washed epithelium was collected by scraping off gently, with a razor blade, the exposed surface of the gut, and was homogenized directly, without passing through the masher.

In saline suspensions, cells and their free constituents were found to undergo definite changes, especially pronounced in nuclei and mitochondria, the latter generally assuming a spherical shape (Claude and Fullam, '45; Claude, '46). Cellular material is best preserved when homogenized in a 0.88 M sucrose solution (Hogeboom, Schneider and Palade, '47 and '48). In this medium, the nuclei appear turgid, structureless, with a life-like, glassy transparency; the mitochondria retain their rod-like, or filamentous shape; the ground substance remains translucent and is apparently gelated, as shown by the absence of any intracellular motion. The appearance of liver, kidney and pancreatic cells in hypertonic sucrose very closely resembles that of the same cells observed directly in tissue fragments obtained after forcing the organs through the masher and examined before addition of any suspension medium. The

only difference noted was the higher refringence exhibited by the mitochondria in 0.88 M sucrose. In this medium, isolated cells and cell organelles can still be stained supravitally with various basic dyes within the first 3 to 4 hours following the preparation of the homogenates. Although dissociation of tissues by homogenization may appear as a drastic procedure, it is clear that cells isolated in this manner are reasonably well preserved especially when suspended in an appropriate medium as 0.88 M sucrose.

The use of cells prepared by tissue homogenization as material for experimental cytological studies presents definite and numerous advantages over the usual histological techniques: (1) it permits a finer dispersion of cellular material than teasing; (2) it offers considerable facility in handling; mounting for observation consists merely in putting a drop of homogenate on a slide and covering it with a coverslip; (3) the action of various reagents on cells and cell structures is more rapid, more uniform, and more direct in homogenates, than in tissue blocks; (4) the effects of the various reagents employed can be observed without delay, avoiding such long and complicated intermediate operations like dehydration, clearing, embedding, sectioning and mounting; (5) the systematic study of the evolution of a given process is made possible, since homogenate preparations can be kept under continued observation during an experiment. The tissue homogenate method has proved to be especially useful for the study of the nature of the Golgi apparatus.

Vital staining. Neutral red, methylene blue and brilliant cresyl blue were employed, dissolved in the same saline or sucrose solution as that used for the respective homogenates. The dyes, usually at 1:5,000 or 1:10,000 dilution, were added to the homogenates at room temperature either in a test tube or between slide and coverslip, in this case, by placing in contact, without mixing, one drop of cell suspension and one drop of the dye solution.

Lipid staining. Sudan Black,[1] Sudan Red, and BZL Blue[2] in 70% ethanol solutions were used for staining lipid inclusions (Lison, '36). The homogenates were stained in test tubes by adding the dye solution drop by drop, under continuous agitation, until suitable concentrations of dye and ethanol were attained. Sudden addition of the stain, resulting in high, local ethanol concentrations, would produce coarse precipitation and would remove from the cells certain lipid inclusions.

Cytological techniques for the demonstration of Golgi apparatus. Various tissues, serving as controls, were treated according to Nassonov's osmium impregnation ('23) and Aoyama's silver impregnation ('29) techniques and the appearance of the Golgi apparatus was compared with that of myelin figures obtained in homogenates.

OBSERVATIONS

Distribution and Nature of Lipid Inclusions in Fresh Material

A. *Hepatic cells.* When observed in small pulp fragments or in saline or sucrose homogenates, fresh liver cells contain as visible inclusions, in addition to mitochondria and nuclei, two different types of highly refringent drops and droplets.

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[1] Sudan Black: George T. Gurr, London; and National Aniline Division.
[2] BZL Blue: George T. Gurr, London; and Ciba, Basle.

The first type is represented by large, colorless drops, variable in size, usually one to 4 μ in diameter. They are found irregularly distributed throughout the cytoplasm (fig. 1). Their number is increased by fasting. Sudan black stains them intensely and they are rapidly blackened if osmium tetroxide solution is added to the homogenates.

The second type is represented by droplets much smaller, more uniform in size, 0.5 to 1 μ in diameter, yellowish in color, and which have a definite distribution. The large majority is found at the periphery of the cell, accumulated close to the bile capillaries, a few being occasionally seen in other parts of the cytoplasm (fig. 1). Their number appears to be greater before, and to decrease after feeding. Although the distribution of these refringent inclusions is not always strictly confined to the regions mentioned, for convenience, the first type will be referred to as central drops, and the second type as peripheral droplets.

In sucrose homogenates, supravital staining of liver cells with neutral red was slow and inconstant. If successful, all the peripheral droplets were colored orange-red. Occasionally a few of the central drops stained similarly. Methylene blue and brilliant cresyl blue concentrated also in some drops and droplets, but the results were never as clear as with neutral red. That the experimental conditions were favorable for supravital staining was indicated by the behavior of the Kupffer cells present, which took the vital dyes rapidly and constantly, and concentrated them conspicuously in large and numerous drops and vacuoles. The peripheral droplets of the liver cells blackened slowly with osmium tetroxide, and stained with Sudan black if the ethanol concentration in the homogenate was slowly and progressively raised to about 30%. With rapidly established ethanol concentrations, they conglomerated in larger droplets, or completely disappeared. When cells already stained supravitally with neutral red were treated under the microscope with Sudan black solutions, rapid and intense blackening of the red drops and droplets could be witnessed.

The peripheral droplets were not appreciably affected by changes in the concentration of the medium, their size and shape remaining apparently unaltered when the cells were suspended in media as different osmotically as hypotonic (0.07 M) saline, or hypertonic (0.88 M) sucrose. Likewise, they were remarkably resistent to autolytic processes, and to variations in H-ion concentration, retaining their appearance when the pH was lowered to 6.0. Under these extreme conditions, they continued to stand out conspicuously as bright, refringent objects in their usual peripheral position. This strikingly contrasted with the behavior of mitochondria, which were highly sensitive to changes in osmotic pressure and H-ion concentrations that quickly caused them to vacuolize, transform into "ghosts," and finally disintegrate. The pH sensitivity of mitochondria was especially marked in isotonic media. It was possible to take advantage of this situation to clear up the cytoplasm, and thus render the peripheral droplets clearly apparent (fig. 1). This is especially useful in the case of hepatic cells,

where mitochondria are so numerous that they interfere with the observation of other inclusions.

The lipid nature of the refringent drops and droplets just described was demonstrated by their affinity for osmium tetroxide and particularly for Sudan black. A difference in the chemical constitution of these two types of lipid inclusions was indicated by differential staining with neutral red. Tests on lipid emulsions of various compositions had shown that the affinity for this dye was proportional to the phospholipid content of the fatty globules. Observations on this point will be exposed in a second paper (Palade and Claude, '49).

From these it can be concluded that fresh liver cells contain two kinds of lipid inclusions, namely: (a) central drops, showing an unconstant affinity for neutral red, and in which, therefore, triglycerides are probably predominant; (b) peripheral droplets, with constant affinity for neutral red, and which can be considered to be composed largely of phospholipids. The fact that these peripheral droplets lack osmotic sensitivity indicates that they have probably a massive structure, or at least a very firm shell. Accordingly, they should be considered as massive droplets, rather than vacuoles. Their typical location, in the neighborhood of the bile capillary, and their accumulation in the periods preceding meals may suggest that these peripheral, predominantly phospholipid droplets are connected with the external biliary secretion of the liver cell.

B. *Other cellular types.* In other cell types, namely in rat kidney, pancreas, intestinal and epididymal epithelium, and in chicken gizzard epithelium and glandular cells, similar refringent drops and droplets, most of them showing affinity for neutral red in supravital staining, were found agglomerated close to the nucleus, on its apical side, in the so-called "Golgi zone" of the cell. Scattered, refringent droplets were encountered in rat, rabbit and chicken nerve cells, and smooth muscle fibers. Under proper conditions, all of them could be stained with Sudan black, and the majority with neutral red. As in the case of the peripheral lipid inclusions of the liver cell, it can be concluded that droplets, supravitally stainable with neutral red, contain phospholipids in high proportion.

Morphological Changes Induced in Lipid Inclusions by Ethanol

Striking transformations in the morphology of the lipid inclusions of the cell were found to follow the addition of ethanol to liver homogenates. Such transformations appeared when the ethanol concentration in a homogenate was raised to, and above, 30% by the progressive addition of a 70% ethanol solution. The changes were most apparent if Sudan black was present in the alcoholic solution used, the dye serving then as an indicator for lipid structures. In a 0.88 M sucrose homogenate, containing 40 to 45% ethanol, the peripheral droplets, if preserved, and some of the central, more stable fat drops began to swell, maintaining for a while their original spherical shapes. Soon, however,

they became irregular by the appearance at their surface of protuberances of various forms. Depending on the number of lipid inclusions which gave rise to them, the cytoplasm was rapidly occupied by a variety of polymorphic bodies, which changed their form slowly and unceasingly (fig. 2). From time to time, some of these bodies were seen to contact and coalesce, thus forming larger and even more complicated figures. These transformations could be observed and followed rather easily if the figures were stained with Sudan black. However, the outline and details of such structures may be obscured by the excessive thickness of certain cells, or by the intervening cytoplasm, which is often colored, although faintly, by the dye. If one of these polymorphic bodies happened to reach under the cell membrane, and succeeded in breaking through, its nature became unmistakable. It was then clearly apparent that the offshoot, now expanding freely in the medium (figs. 3 and 4), was a myelin figure, readily recognizable by its characteristic tubular, or ampular shape, the smoothness of its outlines, and the rigorously constant thickness of its walls.

As is known, a myelin figure is an organized structure, produced by the orderly molecular arrangement of phospholipids, soaps, and other incidentally associated substances. The molecules of myelinizable substances are generally bipolar. In forming myelin figures, they orient themselves into bimolecular films, in which they are apposed by their hydrophobic poles. The walls of a myelin figure are made up of a variable, usually considerable number of such double layers disposed concentrically, between which molecular films of water are regularly interposed (Nageotte, '37; Teitel, '46). As a rule, myelin figures have a central cavity, which can expand by accumulation of water. Such figures present an amazing polymorphism, the causes of which are not completely understood at present. They may appear predominantly vesicular or tubular, depending on their chemical components and the nature of the medium, but myelin figures are essentially unstable structures which, even under given experimental conditions may change one form to another (Leathes, '25; Nageotte, '37).

The observations just described indicate that the polymorphic bodies produced within liver cells, under the influence of ethanol, were true myelin figures developed at the expense of pre-existing lipid inclusions. In the cells, however, the myelin figures were not permitted to expand freely, but were forced to adapt themselves to the spaces available between the masses of precipitated cytoplasm. As a result, and depending on local conditions, the intracellularly developing myelin figures became folded, coiled, and generally packed together into polymorphic bodies. The importance of paths of least resistance in conditioning the position and the shape of such figures, was illustrated by their relations with the nucleus, which acted as a resistant, solid body. When a myelin figure reached the nucleus, as happened frequently, its growth was guided by the nuclear surface, and the result was a cap (fig. 5), or a perinuclear wreath

(fig. 6). This directed growth, which could be watched under the microscope, demonstrated that less resistance was encountered between the nuclear surface and the precipitated cytoplasm, a situation recalling a cleavage space.

As already mentioned, in 0.88 M sucrose homogenates, polymorphic, vesicular figures were most numerous in the presence of 40 to 45% ethanol. In higher ethanol concentrations, namely 45 to 55%, fine tubular figures became predominant and distinct canaliculi (fig. 11, upper cell), more or less twisted and frequently branching, could be seen within practically every cell. An endless variety of forms was thus produced by the haphazard combinations of tubules and capricious dilatations of myelin figures as illustrated in figures 7, 8, 9, 10 and 11. The results obtained in the presence of even higher ethanol concentrations, namely 60 to 65%, were very different, as shown by the appearence of numerous new myelin figures with different locations. At such concentrations, an efflorescence of small, vesicular forms was produced, throughout the entire cytoplasm, without necessary connection with the pre-existent lipid inclusions. Practically every one of the newly formed myelin figures was a hollow sphere, the cavity of which was clearly visible. It is probable that these new myelin figures, scattered in the cytoplasm, arose from phospholipids liberated by the disintegration of lipid-protein complexes of the cytoplasm, especially of the mitochondria and microsomes which have been shown to be particularly rich in phospholipids (Claude, '46).

Intracellularly developed myelin figures had the tendency to migrate through breaks in the cell body, or ruptures of the cell membrane, and to move partially or entirely out of the cell (figs. 3, 4, 9). When observed in cell clumps, they were seen to use the available open spaces, such as bile (fig. 12), and even blood capillaries, wherein they continued to grow and migrate towards the periphery until they reached the border of the clump, where they coalesced with other figures and finally formed huge and complicated structures floating in the medium (fig. 13). When this migration process was completed, only a few small bodies remained scattered inside the cells: they represented myelin figure fragments, or unmyelinizable residues (fig. 13). In some cases, the migrated figures left behind them clear, tortuous spaces in the precipitated cytoplasm (fig. 11, upper cell).

The lipid inclusions which had been liberated mechanically during homogenization developed free myelin figures which, with respect to morphological changes, responded similarly to increasing ethanol concentrations as the intracellular figures, the only difference being that such changes occurred already at somewhat lower concentrations. As already indicated, intracellular and extracellular myelin figures showed a strong and characteristic affinity for Sudan black. They stained also, but more faintly, with other lipid dyes, for instance BZL blue and Sudan red, when these were added beforehand to ethanol.

Morphological transformations, identical to those undergone by the lipid inclusions of liver cells, were encountered in all the other cellular types investigated in this respect. In every case, under the influence of 40 to 55% ethanol, myelin figures appeared, always starting from pre-existing lipid droplets. The spherical, or vesicular forms, which developed at the lowest ethanol concentrations, were progressively transformed into ampules and tubules as this concentration was raised to 55%. The same guiding effect exerted by the surface of the nucleus, and the same tendency of the myelin figures to migrate along paths of less resistance, were encountered in all cells. In the majority of epithelial cells, myelin figures, as a rule, appeared in the apical zone, close to the nucleus, precisely where refringent lipid droplets were normally found in the fresh condition. The figures did not leave this zone, except after a long period of growth. The result was that in renal (fig. 14), pancreatic (fig. 15), intestinal (fig. 17), and epididymal epithelial cells (fig. 18) of the rat, the myelin figures presented a dominant apical position which was rarely reversed. The same apical position, the same relations with the nucleus, and the same growth features were encountered in myelin figures produced in epithelial and glandular cells of chicken gizzard (fig. 16).

The demonstration of myelin figures induced by ethanol in nerve cells was more difficult to achieve because of the abundance of phospholipid material in nerve sheaths, giving rise to innumerable, free myelin figures, which interfered with an accurate observation of the cell bodies. This difficulty was avoided by using brains of 17-day-old chick embryos, in which nerve fibers were incompletely myelinized. In such material, it was possible to produce definite myelin figures in practically every cell, by means of 50 to 55% ethanol. Examples of complicated, tubular, or reticular structures thus produced in pyramidal, and motor pericarya, as well as in the pericaryon of a Purkinje cerebellum cell are illustrated in figures 19, 20 and 21, respectively. More compact forms, localized close to the nucleus, were found in neuroblasts (fig. 22), and the apical disposition, typical for certain epithelial cells, was encountered in ependymal cells (fig. 23), elements which have clearly retained their epithelial character. Ethanol was able to induce myelin figures in other cell types, such as the epithelial cells of bile and pancreatic ducts, the epithelial cells of the kidney pelvis, vascular endothelial cells, fibroblasts and smooth muscle fibers, as they were encountered in various tissue homogenates. Some of these figures were remarkable for their slenderness and their complexity, especially those developed in the smooth muscle fibers of arterioles (fig. 26) and of the gizzard muscularis (fig. 25).

In the observations so far reported, the determining factor in the production of intracellular myelin figures was the ethanol concentration in the medium, but other adjuvant factors were found to be also involved in this process. This was the case with respect to salt and sucrose concentrations and the acidity of the medium. In 0.88 M sucrose, for example, localized myelin figures began to

form in over 40% ethanol, and a general efflorescence was produced in 60 to 65%. With 0.07 M NaCl, figures appeared, and a general efflorescence occurred in ethanol concentrations as low as 30 and 50%, respectively. There were appreciable differences even between isotonic solutions of saline and sucrose, myelin figures beginning to appear close to 40% ethanol in 0.25 M sucrose, as compared with 35% in 0.15 M NaCl. In this test, sucrose appeared to delay the formation of myelin figures, suggesting that it may have a stabilizing effect on phospholipids, possibly by reducing their solubility. An increase in temperature, by keeping the preparations at 38°C., accelerated the formation of the figures, and favored the occurrence of complicated forms. A lowering of the pH, as spontaneously produced by atmospheric CO_2 at the periphery of unsealed preparations, brought up complicated figures even in ethanol concentrations as low as 30%, in 0.88 M sucrose. The instillation of weak solutions of HCl between slide and coverslip in a homogenate mount produced a spectacular speeding up in the formation of complicated tubular figures, from more simple forms. Such effects could be followed closely since the incoming acid wave in the microscope field was clearly indicated by the changing color of Sudan black from deep blue to purple-black. Desiccation likewise had a definite effect in speeding up the formation of myelin figures in the cellular material of the homogenates.

DISCUSSION

The observations related in this paper show that myelin figures can be produced in a variety of vertebrate somatic cells by means of ethanol in given concentrations. The myelin figures develop at the expense of pre-existing inclusions which appear in living or fresh cells as highly refringent droplets. All these droplets can be stained in tissue homogenates by Sudan black and most of them show an affinity for neutral red and other basic dyes commonly used in supravital staining. The sudanophily shows that these refringent droplets are composed of lipids, while their affinity for neutral red and the ability to form myelin figures indicate that they contain phospholipids in large proportion. The intracellular myelin figures produced in the manner described exhibit a remarkable polymorphism, partially conditioned by the amount of ethanol in the medium. For a certain range of ethanol concentrations, generally comprised between 40 and 55%, the morphology and topography of the intracellular myelin figures are surprisingly similar to those assumed by the Golgi apparatus in corresponding cells. This striking similarity will be discussed in the following pages in relation with the various cell types investigated.

In hepatic cells, the Golgi apparatus has been described as a polymorphic structure (Nassonov, '26; Dornesco, '30; Richardson, '34; Solé and de Robertis, '35; Tarao, '39), and a secretory cycle, in connection with bile production, has been ascribed to it (Cramer and Ludford, '26). This cycle is supposed to begin with a "resting phase," during which the apparatus assumes the form of a dense,

juxtanuclear network. Structures identical in appearance can be produced by myelin figures which were guided during their growth by the nuclear surface, and consequently appear as juxtanuclear caps or perinuclear wreaths (figs. 3, 4, 5 and 6). When the "ascending phase" of the cycle reaches its climax, the apparatus shows a peribiliary distribution and may be represented by a number of canaliculi, some of them "connected" with the bile capillary. Figures 7 and 8 show that this peripheral distribution can be duplicated by myelin figures which frequently exhibit clear canalicular forms (fig. 11, upper cell). In this peribiliary location, the Golgi apparatus may assume at times a peculiar aspect, if cells happen to be observed frontally. In this case, it appears as a network restricted to a narrow band extending the full length of the cell in continuity with similar structures in neighboring cells (Nassonov, '26). Under similar conditions of observation, such aspects can be duplicated by experimentally produced myelin figures (fig. 9). Between these two extreme phases of the cycle, namely the "resting phase" with the juxtanuclear disposition, and the "discharge phase" with the corresponding peribiliary location of the apparatus, intermediate stages have been described; likewise, innumerable intermediate appearances can be produced by various combinations of myelin figures (fig. 10). The bile capillaries are almost always impregnated in liver preparations treated according to the Golgi apparatus techniques (Dalton, '34; Deane, '44). This fact, generally overlooked, may correspond to the presence in bile capillaries of migrating myelin figures, as clearly illustrated in figure 12. Lipid residues left in cells after the migration of myelin figures has taken place (fig. 13) could be compared to the "Golgi remnants," which are supposed to represent the last phase of the secretory cycle. The clear spaces left behind in some cells by migrated myelin figures (fig. 11, upper cell) are reminiscent of the negative images of the Golgi apparatus. The myelin figures which develop at the expense of peripheral droplets strongly recall the Golgi apparatus during the "ascending phase" of its cycle when it assumes a peribiliary disposition. The juxtanuclear myelin forms, derived from the central drops, irregularly distributed in the cytoplasm, correspond to the aspects of the apparatus in its "resting phase" and at the beginning of its secretory cycle.

In the case of other cellular types, the parallelism between Golgi apparatus and intracellular myelin figures is even more striking. As is known, the mammalian liver cell does not exhibit a clear-cut "polarization," and has no definitely localized "Golgi zone." In epithelial and glandular cells, where such a zone exists, however, for example in the kidney (fig. 14), pancreas (fig. 15), gizzard mucosa, intestine (fig. 17), epididymis (fig. 18), the typical topography of the Golgi apparatus is faithfully reproduced by that of the myelin figures arising from the lipid droplets originally located in the corresponding "Golgi zones." In such cells, the myelin figures, like the Golgi apparatus, assume an apical polarization, only exceptionally reversed. In the pericarya of nerve cells, both

the artificially produced myelin figures and the Golgi apparatus present similar features. Both formations appear as intricate and voluminous networks, or separate bodies, scattered through the entire mass of cytoplasm, without any preferential localization (figs. 19, 20 and 21). In neuroblasts, myelin figures, as the Golgi apparatus, have generally a more concentrated, juxtanuclear disposition (fig. 22), and in ependymal cells apical polarization of both is re-encountered (fig. 23).

The fine structural details described as characteristic for the Golgi apparatus can also be duplicated by intracellular myelin figures. Structures with a peripheral, chromophile, or osmiophile shell, surrounding a chromophobe, or osmiophobe mass, as demonstrated in Richardson's illustrations ('34), can be reproduced by myelin figures, as shown in figures 3 and 4. In this case, the lipid wall of the figure corresponds to the "Golgi Externum" while the cavity corresponds to the more transparent "Golgi Internum." Vesicular figures, or localized swellings, as shown in figures 19, 20 and 22 (upper cell), are similar to what is known in Golgi apparatus terminology as "mono-systems." Images of agglomerated vesicles, or foam structures, as illustrated in figures 11 (lower cell), 16, 18 (lower cell), and 19, duplicate the so-called "poly-systems." The reticular aspect, stressed in the original description of Golgi, but less and less emphasized in later literature, can be reproduced by the branching of tubular figures as shown in figures 25 and 26, and, even more frequently, by the superimposition of the loops of one or more twisted and coiled tubules (fig. 10). The space available for the expansion of growing myelin figures is the factor responsible for the formation of networks. Figure 24 shows three different myelin forms developing around the same nucleus: the first on the right side is contained in a mass of cytoplasm, and appears as a compact, irregular body; the second appearing under the nucleus is growing in a more yielding environment, and has formed a loosely entangled heap which, in projection, appears as a network; the third on the left side, floating freely in the medium, has developed as a straight tube.

As shown in the preceding pages, myelin figures can duplicate faithfully the numerous and different forms ascribed at various times to the Golgi apparatus. Thus they can take the appearance of massive, or canalicular networks, scattered strands, canaliculi, polymorphic bodies, "poly-systems" and "mono-systems." Images similar to those of the "systems" are frequently produced by intracellular myelin figures. Their lipid walls correspond to the chromophilic shell, while their cavities, appearing as clearer central regions, duplicate the chromophobic core of a "system." As is known, this "duplex structure" appearance has been considered as typical for the Golgi apparatus since Hirschler ('18; '27) and Hirsch ('39). Likewise, the intracellular topography of myelin figures very closely parallels that of the Golgi apparatus in various cellular types. Both structures exhibit a definitely dominant apical localization in polarized epithelial cells, and show a more dispersed and irregular distribution in

cellular elements lacking a clear polarization such as liver cells and nerve cell pericarya. The volume of the two structures shows also parallel variations from one cellular type to another. Both are found as conspicuous appearances in glandular cells and in nerve cell pericarya and as delicate formations in fibroblasts and smooth muscle fibers.

These facts strongly suggest that the Golgi apparatus may be a myelin figure or a complex of myelin figures artificially induced in cells by given cytological techniques. Such a hypothesis could reconcile the different aspects presented by cells of the same type when examined in the fresh condition or after the application of recognized cytological procedures. This hypothesis could be tested by investigating the possible production of intracellular myelin figures under the influence of the most commonly used Golgi apparatus procedures. This point will be the subject of the second paper of this series.

SUMMARY

1. Intracellular myelin figures have been produced experimentally in a variety of vertebrate somatic cells, including glandular epithelia from liver, kidney, pancreas and chicken gizzard, epithelia from intestine and epididymis, nerve cell pericarya, fibrocytes, and smooth muscle fibers.

2. The myelin figures were produced by adding ethanol to the medium in favorable concentrations, namely 40 to 55%.

3. The intracellular myelin figures were found to develop at the expense of the lipid inclusions pre-existing in the fresh cells in the form of refringent droplets. The lipid nature of these cell inclusions was demonstrated by their affinity for Sudan black. Their ability to concentrate neutral red, and to form myelin figures indicates that these inclusions contain phospholipids in appreciable proportion.

4. The myelin figures induced intracellularly by ethanol were found to duplicate faithfully, in morphology and topography, the Golgi apparatus of corresponding cells.

5. The results suggest that the Golgi apparatus is a myelin figure, or a complex of myelin figures, artificially induced in cells during the preparation of the cytological specimens.

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EXPLANATION OF PLATES

All photomicrographs were taken on Kodak M plates, with a Bausch and Lomb research microscope and camera, using an apochromatic immersion objective (2 mm; 1.20; × 90) and compensated ocular (× 12.5), and a Kodak Wratten filter No. B-58. The photomicrographs were taken at 1100 ×, and enlarged to 1800 ×.

PLATE 1

EXPLANATION OF FIGURES

1 Isolated rat liver cell. (Liver homogenate in 0.25 M sucrose, buffered at pH 6.0, and kept 4 hours at 4°C.) Two types of lipid inclusions stand out after the cytoplasm has been cleared of mitochondria by lowering the pH to 6.0: (a) fat drops which can be seen irregularly distributed throughout the cell body; (b) peripheral lipid droplets which, in this case, are especially abundant along the right border of the cell. The inclusions are bright or dark, depending on their position with respect to the focal plane.

2 Two rat liver cells. (Liver homogenate in 0.88 M sucrose; ethanol added to a final concentration of 45%; Sudan black.) The preparation illustrates the effect of ethanol at this concentration upon the lipid inclusions, which appear swollen in the lower, and already transformed into polymorphic bodies, in the upper cell.

3 Ruptured rat liver cells. (Liver homogenate in 0.25 M sucrose; 45% ethanol; Sudan black.) The preparation illustrates the perinuclear development of a tubular myelin figure, mostly embedded in the cytoplasmic mass. An end expanded freely in the medium is readily recognizable as a myelin figure. The intracytoplasmic part of this figure is comparable to the Golgi apparatus as it appears in Richardson's illustrations ('34).

4 Ruptured liver cell. (Liver homogenate in 0.25 M sucrose; 45% ethanol; Sudan black.) The picture illustrates a juxtanuclear myelin figure in the process of migrating out of the cytoplasmic mass. The free portion exhibits unmistakably the characteristics of a myelin figure. The juxtanuclear cap shows clearly that the walls of the figure correspond to the chromophile, and the cavity to the chromophobe of a Golgi "mono-system."
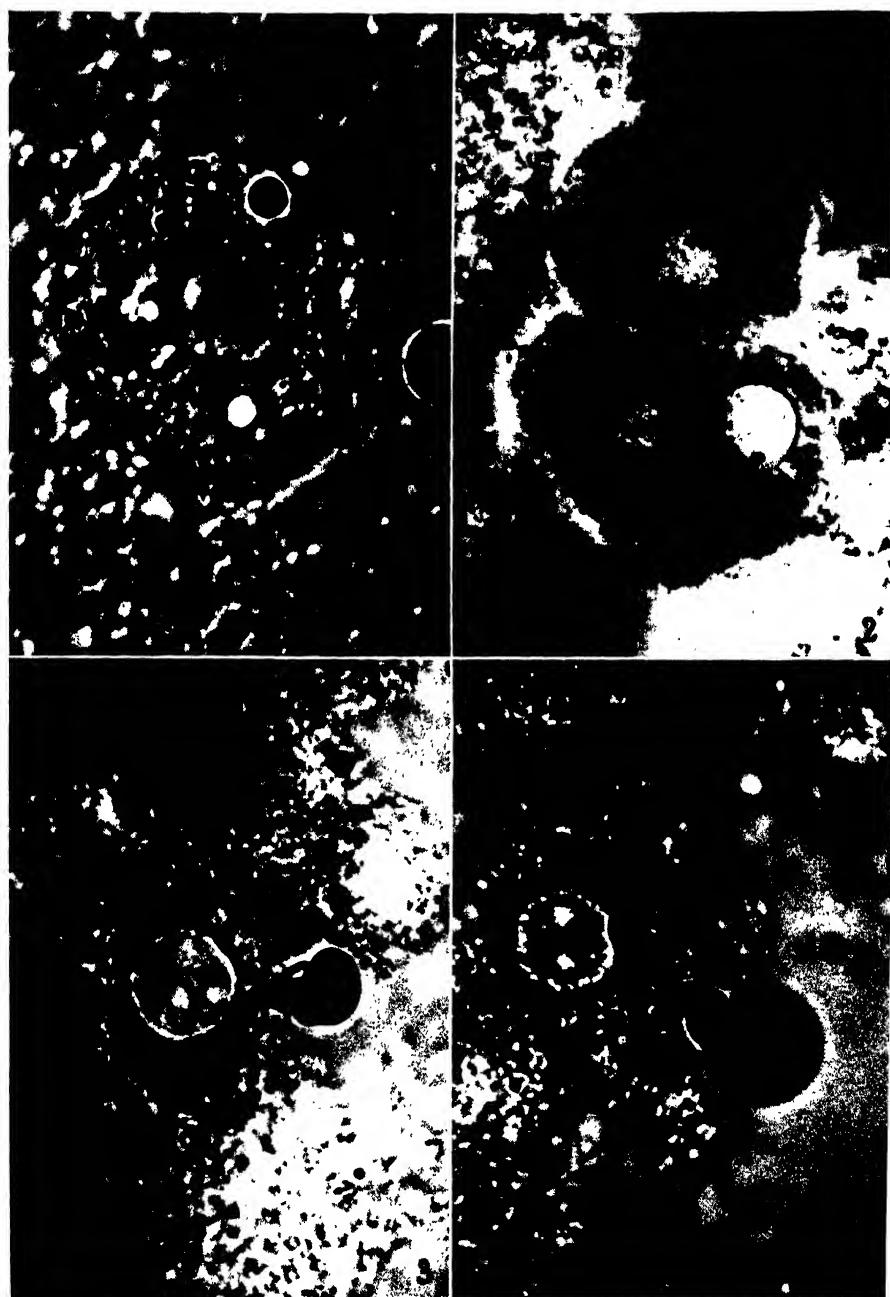
5 Rat liver cell. (0.25 M sucrose homogenate; 45% ethanol; Sudan black.) The picture shows intracellular myelin figures, forming a perinuclear cap. Other myelin figures can be seen in the lower part of the cell, and along the left border. An extracellular figure, out of focus, appears as a shadow on the upper end of the cell.

6 Group of rat liver cells. (0.25 M sucrose homogenate; 45% ethanol; Sudan black.) The picture shows intracellular myelin figures disposed in perinuclear wreaths. Some peripheral figures are also present. The tubular end of one of them has migrated out in the medium and appears as an elongated body, partially out of focus, perpendicular to the border of the upper cell. Figures 5 and 6 illustrate the guiding effect of the nuclear surface upon growing myelin figures. Such perinuclear structures develop generally at the expense of central fat drops.

7 Rat liver cells. (0.25 M sucrose homogenate; 45% ethanol; Sudan black.) The picture shows groups of peripheral myelin figures, accumulated along the cell borders and duplicating the peribiliary disposition of the Golgi apparatus during the "discharge phase" of its cycle. Such myelin figures developed at the expense of the peripheral lipid droplets.

8 Group of rat liver cells (0.88 M sucrose homogenate; 55% ethanol; Sudan black.) The picture shows intracellular myelin figures, some of them disposed along a bile capillary (right center). The peribiliary myelin figures developed at the expense of peripheral lipid droplets.

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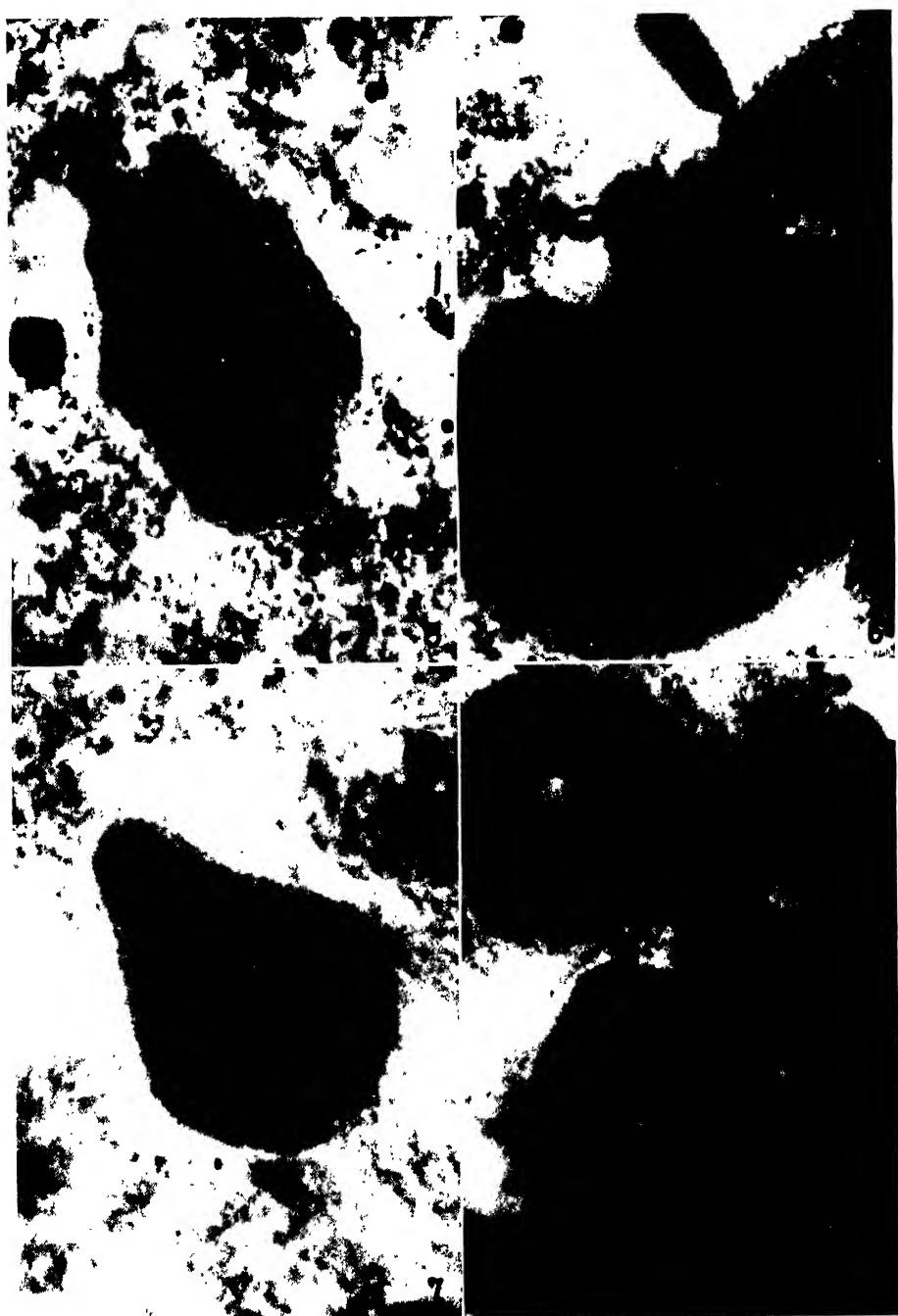
9 Two adjacent rat liver cells. (0.25 M sucrose homogenate; 45% ethanol; Sudan black.) The boundary between the two cells is not visible in this optical section, and the nucleus of the upper cell, located in the upper half of that cell, is not in focus. The picture shows contiguous myelin figures forming what appears as a continuous band along the right border of the cells. This arrangement duplicates the particular distribution of the Golgi apparatus described by Nassonov ('26). A slender tubular myelin figure is seen migrating from the upper cell.

10 Two rat liver cells. (0.25 M sucrose homogenate; 45% ethanol; Sudan black.) The lower cell contains numerous, scattered myelin figures which, by the superposition of their loops, create the appearance of an intracellular network, in this case partly out of focus.

11 Two rat liver cells. The upper cell (liver homongenate in 0.88 M sucrose; 55% ethanol; Sudan black) shows tubular myelin figures moving towards the lower margin. They could be compared with the intracellular bile canaliculi supposed to represent the Golgi apparatus in Cramer-Ludford drawings ('26). The clear space in the lower left quadrant of the cell is a cavity previously occupied by a myelin figure, which later migrated out of the cell. The lower cell (liver homogenate in 0.25 M sucrose; 45% ethanol; Sudan black) shows peripheral polymorphic bodies, those in the upper right quadrant duplicating a "poly-system."

12 Group of liver cells. (0.25 M sucrose homogenate; 45% ethanol; Sudan black.) The picture illustrates the migration of myelin figures through a bile capillary. Myelin figures originating in two adjacent cells have penetrated the bile capillary and use its lumen for their progression.
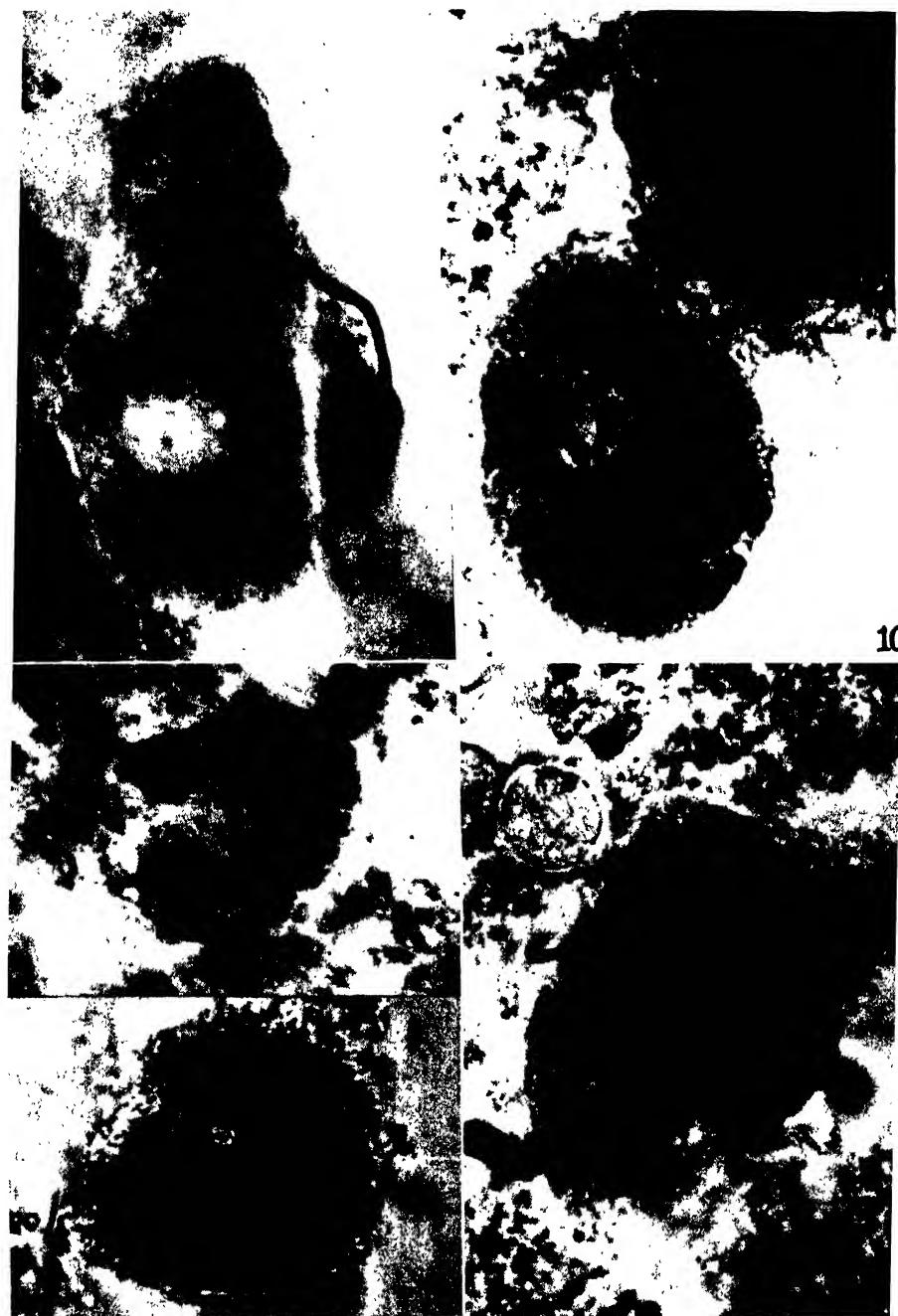
13 Margin of a clump of liver cells. (0.25 M sucrose homogenate; 45% ethanol; Sudan black.) The picture shows huge, sessile myelin figures floating freely in the medium. Their cavities, the smoothness of their outline, the uniform thickness of their walls, as well as their polymorphism (tubules, vesicles, ampules) are clearly apparent. Residues of myelin figures left in the cells, especially along the cell margins, duplicate the so-called "Golgi remnants."

14 Rat kidney. (0.88 M sucrose homogenate; 55% ethanol; Sudan black.) The picture represents an optical section through the proximal convoluted tubule of a nephron (pars convoluta portionis principalis). The cell boundaries and the brush border are not visible. The intracellular myelin figures, like the Golgi apparatus of the respective cells, exhibit a dominant apical, juxtanuclear location.
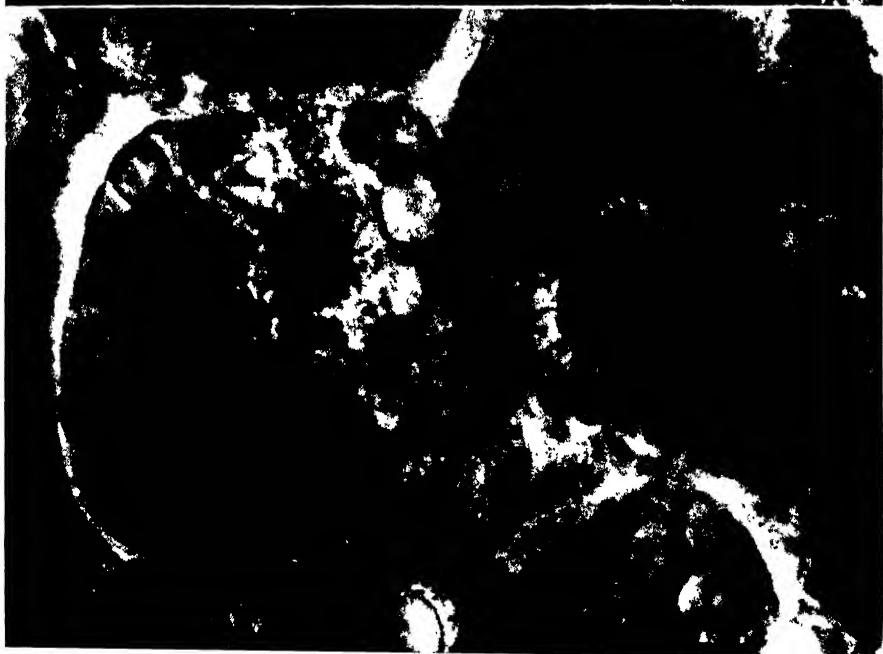
15 Two acini of rat pancreas. (0.88 M sucrose homogenate; app. 50% ethanol; Sudan black.) The upper acinus, with only two cells visible, shows a peripheral myelin figure complex. The lower acinus shows distinctly three cells with myelin figures developing at the apical side of their nucleus, i.e., in the typical "Golgi zone" of pancreatic cells. A 4th cell of the same acinus, also containing apical myelin figures, is out of focus. A myelin figure can be seen in the glandular lumen at the confluence of the two acini.

16 Keratin gland from chick gizzard mucosa. (0.88 M sucrose homogenate; app. 50% ethanol; Sudan black.) The picture shows a lateral view of a glandular tube, the lumen of which is not visible. The nuclei of the glandular cells are conspicuous. These nuclei are surrounded by numerous myelin figures, which appear as perinuclear bodies or caps. The Perinuclear cap at the center of the picture is especially extensive. Other myelin figures, showing clear central cavities, duplicate the so-called "poly-systems" (left of the center with lighter outlines), or "mono-systems" (close to the first nucleus below the "poly-systems").

17 Epithelial cells from rat intestine. (0.88 M sucrose homogenate; 42% ethanol; Sudan black.) In the 4 cells shown, myelin figures have developed on the apical side of the nuclei, i.e., the usual "Golgi zone" of these cells. In the upper and lower left cells, the myelin figures are in close contact with the nuclei which partially directed their growth; in the lower left cell, the myelin figure has partially migrated out and its free end has started to expand in the medium.

18 Epithelial cells from rat epididymis. (0.88 M sucrose homogenate; app. 50% ethanol; Sudan black.) The two cells shown contain myelin figures developed on the apical side of the nucleus. The upper cell contains three polymorphic bodies; the lower cell shows a group of vesicular myelin figures, duplicating a so-called "poly-system." The lines crossing the field are sperm tails not in focus.

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16
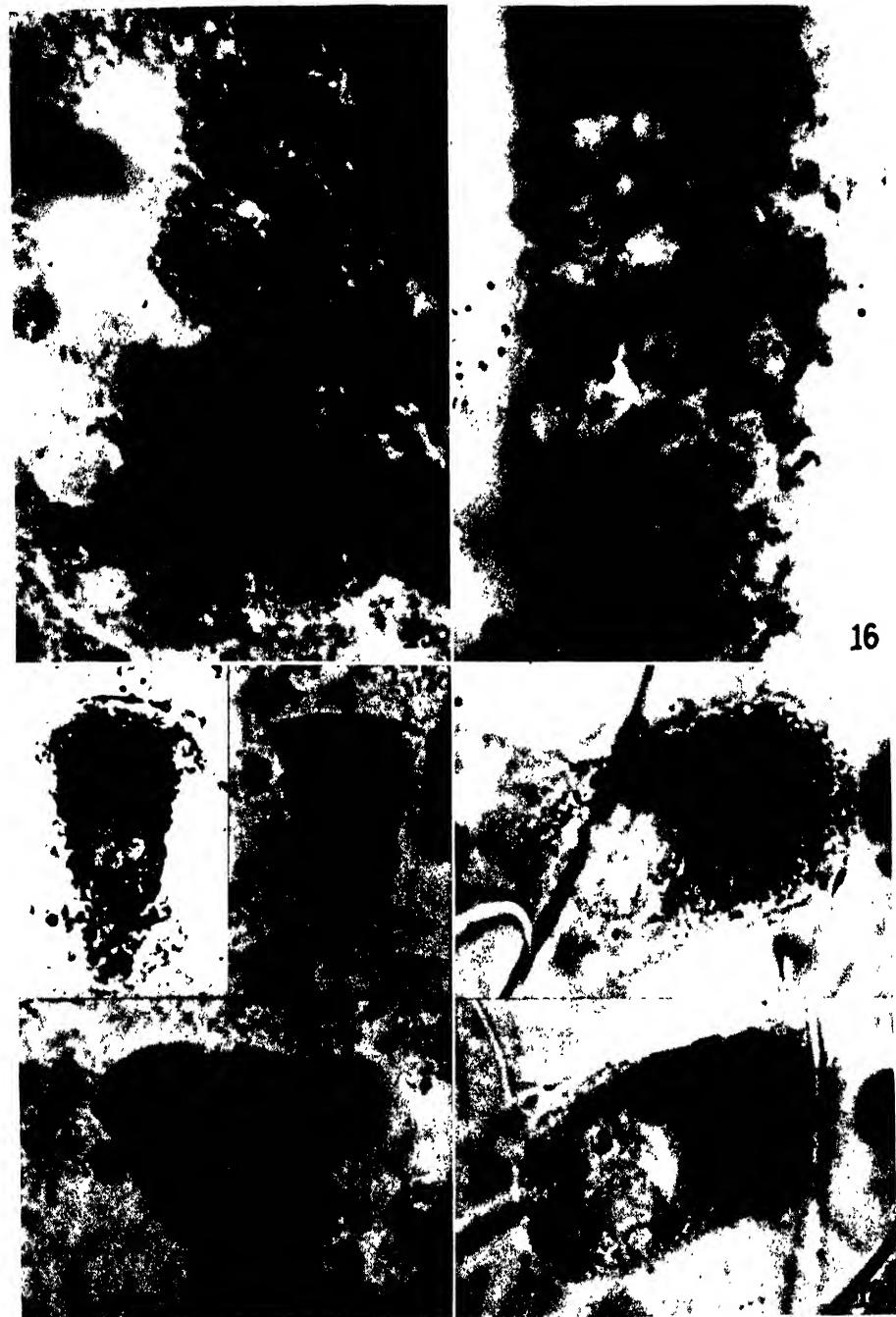
PLATE 6

19 Pericaryon of a pyramidal cell from chick embryo brain. (0.88 M sucrose homogenate; 52% ethanol; Sudan black.) The three main dendrites are visible, but the axone is not in the focal plane. The picture shows numerous intracellular myelin figures, cavities being clearly apparent in most of them. A figure close to the upper pole of the nucleus duplicates a "poly-system," whereas several figures in the lower right quadrant of the pericaryon reproduce so-called "mono-systems." The tip of an elongated myelin figure protruding out of the cell is somewhat out of focus.

20 Pericaryon of a large motor cell from chick embryo brain. (0.88 M sucrose homogenate; 52% ethanol; Sudan black.) The pericaryon contains complicated networks produced by the branching and superposition of tubular myelin figures; parts of the networks are out of focus. A simple, vesicular figure, recalling a "mono-system," can be seen immediately under the nucleus. In the lower left corner of the picture, a small, ampular myelin figure, with a visible cavity, appears to have left the pericaryon.

21 Pericaryon of a Purkinje cell from chick embryo cerebellum. (0.88 M sucrose homogenate; 52% ethanol; Sudan black.) The stump of the axon is still visible slightly on the right of the lower pole of the pericaryon. The stem of the main dendrites appears as an extension of the upper pole. Intracellular myelin figures form a network, parts of which appear close to the nucleus, and along the cell margin. Other portions of the network are not in the focal plane. The cell contains also a few scattered, vesicular myelin figures.

22 Neuroblasts from chick embryo brain. (0.88 M sucrose homogenate; 52% ethanol; Sudan black.) The two cells illustrate compact forms of myelin figures developed against or close to the nuclear membrane. They duplicate the juxtanuclear, compact forms ascribed to the Golgi apparatus of such cells. The upper cell contains also a few simple, vesicular myelin forms, showing clearly apparent cavities, and recalling the "mono systems" with their chromophilic shell and chromophobic core.

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PLATE 7

23 Ependymal cell from chick embryo brain. (0.88 M sucrose homogenate; 52% ethanol; Sudan black.) The cilia and the internal process of the ependymal cell are clearly visible. Myelin figures are developing at the apical side of the nucleus, duplicating the appearance of a polarized Golgi apparatus.

24 The role of environment in conditioning the appearance of myelin figures. (0.25 M sucrose homogenate; app. 45% ethanol; Sudan black.) The same ruptured rat liver cell gave rise to three different, juxtanuclear myelin figures. The upper right figure grew in a mass of precipitated cytoplasm, and appears as a polymorphic body. The lower left figure, developing more freely, produced a coiled tubule which, in optical projection, has the appearance of a network. The upper left figure, floating freely in the medium, gave rise to a straight tube.

25 Muscularis from chicken gizzard. (0.88 M sucrose homogenate; app. 50% ethanol; Sudan black.) The center of the picture shows myelin figures forming a wreath around the nucleus of a smooth muscle fiber. Above this an intricate network, partially extracellular, was produced by the coiling and branching of a myelin figure.

26 Arteriole from rat kidney. (0.88 M sucrose homogenate; app. 50% ethanol; Sudan black.) The lateral view of the arteriole shows delicate myelin figures developing around the nuclei of smooth muscle fibers. At the center of the picture, myelin figures developing over the left pole of a nucleus have the appearance of a typical Golgi network.

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THE NATURE OF THE GOLGI APPARATUS

II. IDENTIFICATION OF THE GOLGI APPARATUS WITH A COMPLEX OF MYELIN FIGURES*

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FOURTEEN FIGURES

In a preceding paper (Palade and Claude, '49) it has been demonstrated that myelin figures can be induced by means of ethanol in a variety of vertebrate somatic cells. Such figures have been found to exhibit a morphology and an intracellular distribution closely similar to those ascribed in the classical cytological literature to the Golgi apparatus of the corresponding cells. These observations suggest that the Golgi apparatus might be a collection of intracellular myelin figures artificially produced during the preparation of the cytological specimens. In order to test this hypothesis it is necessary to find out whether myelin figures develop in cellular material while being treated according to the procedures recommended for the demonstration of the Golgi apparatus. Generally, these procedures comprise two main, successive stages: (a) the fixation, for which a variety of aqueous fixative mixtures have been proposed, and (b) the impregnation which is performed by means of osmium tetroxide, or silver nitrate solutions. In such techniques, however, ethanol does not intervene except after impregnation. Since at that time the apparatus is already present in the cells (Nassonov, '23), its production cannot be ascribed to ethanol. At this point, it may be recalled that Golgi himself ('08), in a modification of his original procedure, proposed the use of an ethanol mixture (32% final ethanol concentration) as a fixative, and recommended it specifically for its better, quicker, and more constant results in the demonstration of the apparatus. Likewise, Addison ('29) suggested the addition of either ethanol or methanol to the fixative of da Fano, in order to insure the appearance of network structures in those materials in which the original method showed only rod-like or granular bodies. Nevertheless, the use of ethanol in fixative mixtures has not prevailed in current Golgi apparatus techniques. If the apparatus is really a collection of intracellular myelin figures, it should be possible to demonstrate that such figures are produced also under the influence of fixatives or impregnating solutions used in the ordinary Golgi apparatus procedures. With this problem in view, experiments were carried out in order to investigate the possible effect on myelin figure formation of: (a) fixative mixtures, such as those of Nassonov, da Fano, and Aoyama; (b)

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OsO$_4$ impregnation solutions; and (c) certain H-ion concentrations similar to those brought about in tissues during fixation with the mixtures just mentioned. Under the conditions of these tests, it was found that myelin figures were regularly produced in cellular material by the action of the mentioned fixatives. Following subsequent osmic impregnation, the intracellular myelin figures so induced appeared indistinguishable from the Golgi apparatus of the corresponding cells.

In a number of cases it was possible to ascertain that such myelin figures developed at the expense of lipid inclusions present as refringent droplets in the fresh material. In order to provide further evidence concerning the relation between myelin figures and cellular inclusions visible in vivo, two different series of experiments on models were carried out. One concerns the effect of Golgi apparatus techniques upon lipid droplets of known chemical composition. The other deals with the effect of the same techniques upon various cellular fractions isolated from tissue homogenates by differential centrifugation. These experiments demonstrated that lipid droplets with a high phospholipid content develop into myelin figures when subjected to the conditions provided by the current Golgi apparatus techniques. Indications concerning the behavior of the lipid inclusions in supravital staining were also obtained.

Materials and Methods

Materials

Rat liver pulp and rat liver homogenates were used in all these experiments.

Methods

Tissue homogenates. The cellular suspensions were prepared as shown in a previous paper (Palade and Claude, '49) by homogenizing liver pulp in various suspension media, namely 0.88 M sucrose, 0.25 M sucrose, 0.15 M NaCl and 0.07 M NaCl.

Technique for the study of the action of fixatives. Small fragments of liver pulp were crushed between slide and coverslip. By applying appropriate pressure, it was possible to obtain thin discs of tissue, suitable for direct microscopic observation. The coverslips were secured by means of vaseline at each corner, and the preparations immersed in jars containing one of the following fixative mixtures: (a) Nassonov, (b) Aoyama, and (c) da Fano, each known to be especially appropriate for the demonstration of the Golgi apparatus. Under these conditions, the fixative penetrates between slide and coverslip, surrounds the edge of the disc, and diffuses into the tissue as it would in a tissue block. Diffusion of the fixative, and its effects on cells were followed by repeated microscopic inspection of the discs. Since capillarity interferes with rapid penetration of the fixatives, large discs, almost reaching the border of the coverslips, were found preferable.

H-ion concentration experiments. As a rule, the fixatives employed for the demonstration of the Golgi apparatus are strongly acid. Accordingly, the effects of various H-ion concentrations on the morphology of cytoplasmic inclusions were investigated.

MacIlvain's citric acid-sodium phosphate, sodium phthalate-sodium hydroxide, and Sörenson's phosphate mixtures, ranging between pH 3.0 and 7.7, were used in these tests. Homogenates were buffered by adding slowly, under continuous agitation, the respective buffer solutions to a final concentration of 0.13 M to 0.15 M.

Models. Phospholipid emulsions, namely 2% soya bean lecithin[1] in distilled water, or 0.15 M saline, were prepared in a 150 cm³ Waring blendor, the blades revolving at low speed for two minutes, then at high speed for the next three minutes. Mixed emulsions were prepared in the same manner, using 2 gm of soya bean lecithin and 1 to 2 gm of olive oil per 96 to 97 cm³ saline or distilled water. The staining of these emulsions by means of vital dyes was tested by mixing on the slide, or in the test tube, equal volumes of the original emulsions with 1:1:000 aqueous solution of neutral red, methylene blue, or Nile blue sulphate. The first two dyes were used also in saline at the same concentration.

In order to test the effect of fixation and impregnation on phospholipid emulsions, these were incorporated in gelatine blocks by mixing a few drops of emulsion with 30% gelatine solution in 0.15 M NaCl at 38°C.; the preparations were allowed to gel and harden at 4°C., for 12 to 24 hours. Small fragments, $2 \times 2 \times 3$ mm, cut from the gelatine blocks, were treated thereafter according to the usual Nassonov technique, i.e., 24 hours fixation in Nassonov mixture followed by washing, then impregnation in 2% osmium tetroxide solution, at room temperature, for periods not exceeding 3 to 4 days. Longer treatment with osmium tetroxide solutions would render the gelatine brittle and would interfere with subsequent sectioning. At the end of the impregnation, the gelatine blocks were washed in running water for 12 to 24 hours, carried through alcohols and methyl salicylate, included in tissuemat, cut at 3μ and mounted on slides, in cedar oil, after passing through xylols.

Experiments with cell fractions. The formation of myelin figures at the expense of cell constituents, separated by differential centrifugation, was investigated on isolated lipid droplets, mitochondria, and microsomes resuspended in water, saline, or sucrose. The separation of the various fractions was performed according to methods previously described (Claude, '46; Hogeboom, Schneider and Palade, '48).

Cytological techniques for the demonstration of Golgi apparatus. As control, small blocks of liver tissue were treated according to Nassonov's osmium impregnation ('23) and Aoyama's silver impregnation ('29) techniques and the appearance of the Golgi apparatus thus demonstrated was compared with that of myelin figures obtained in homogenates and disc preparations.

OBSERVATIONS

1. Morphological Changes Induced by Golgi Apparatus Techniques

A. Effects of Golgi apparatus fixatives. The first attempt to study the effect of Golgi apparatus fixatives on myelin figure formation was made by homogenizing directly the tissue pulp in the mixtures of Nassonov, Aoyama or da Fano, using 1 gm of liver pulp to 5 or 10 cm³ of fixative. In view of the fact that,

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[1] Vegetable Lecithin from soya bean. Amend Drug and Chemical Co., Inc., New York.

at the beginning of fixation, fixatives are appreciably diluted inside tissue blocks by the amount of tissue present, the same mixtures were tested in progressive dilutions using 1 volume of fixative to 1 to 4 volumes of liver homogenate in saline or sucrose solutions. The tissue suspensions were then examined at regular intervals in the course of several hours. Similar tests were carried out with OsO^4 impregnating solutions, either by homogenizing directly liver pulp in 1 or 2% aqueous OsO_4, or by treating liver homogenates with such solutions in final concentrations ranging between 0.1 and 1%. The results of all these tests remained entirely negative, in that myelin figures failed to appear in any of the preparations.

It was then realized that, in the tests just described, the cells were brought in contact with the test solutions almost at once, a situation unlike that prevailing in tissue blocks in which the components of the fixative or impregnation mixtures are known to diffuse relatively slowly. It appeared, therefore, necessary to duplicate as closely as possible the conditions existing in tissue blocks, yet devise ways by which the cells treated could be kept under constant observation throughout fixation and impregnation. To this end, use was made in the following tests of liver pulp in disc mounts, the preparation of which has been described in another part of this paper. Such disc preparations were immersed without delay in suitable fixative mixtures and subjected thereafter to periodical microscopical examination. An interesting fact illustrated by this method has been the demonstration that the various components of the fixative mixtures do not act on the cells together, but in sequence, depending on their respective rate of diffusion in the cellular mass. The results of this dissociated action on cells will be described presently.

Nassonov fixative. Examination of liver disc preparations immersed in Nassonov's showed that the tissue at the periphery was fixed rapidly, but that the subsequent diffusion of the various components of the mixture was soon dissociated, each moving at a different rate towards the center of the preparation. Within 15 to 30 minutes following immersion in the fixative, a definite pattern was established as follows: (a) The outer rim of the preparation, a few cell layers thick, was first and immediately affected. In this region which appeared brown, probably due to osmium dioxide, the cells seemed relatively well fixed, with mitochondria still detectable, although altered. The central lipid drops scattered throughout the cytoplasm retained their spherical shape, and blackened rapidly, while the peripheral lipid droplets, along the bile capillaries, were well preserved, and turned brown progressively. (b) Within this outer brown ring, a second yellow ring appeared and kept enlarging continously towards the center of the disc. Its color was visibly due to the presence of chrome anions, diffusing more rapidly than osmium tetroxide molecules. In this second zone, the lipid inclusions had not yet been reached and fixed by osmium oxides; they appeared swollen and irregular in shape, suggesting that they were already

engaged in slow myelin figure formation. (c) Even more centrally, a third ring could be detected, progressing rapidly towards the interior. This ring was colorless, indicating that chrome anions were still rare or absent in the region. Cellular alterations in this zone indicated that the cells were subjected to increasing acidification. Close to the yellow ring, where the acidity was probably the highest, mitochondria could not be recognized, the cells being occupied by a coarse granular precipitate. In positions more centrally located, the mitochondria appeared less and less damaged, first as "ghosts," i.e., large, pale vesicles, then as distinct more or less swollen granules, and finally, in the center of the disc, not yet reached by any component of the fixative, as well preserved, rod-like bodies. Observations on buffered cellular suspensions in isotonic media have shown that these successive changes occur within narrow ranges of H-ion concentration namely: precipitation of the cytoplasm at pH 5.8; ghost formation below pH 6.5, and swelling of the granular mitochondria below 6.7. Therefore it could be concluded that the changes observed in this third ring were the result of the more rapid diffusion of the H-ions present in the fixative mixture. The lipid inclusions were found to be affected, i.e., swollen and of irregular shape, only in the most acid part of the ring. Within two to three hours following the first contact with the fixative, the swollen lipid droplets developed gradually into definite intracellular myelin figures. These continued to increase in number and complexity during the following 24 hours. At that time, they were found more numerous and complex in the intermediate yellow zone (figs. 1 and 2), less numerous, and simpler in the most acid parts of the acid ring.

Aoyama and da Fano fixatives. As in the case of the Nassonov mixture, myelin figures likewise were found to develop in tissues treated with the fixatives recommended by both Aoyama and da Fano for the demonstration of the Golgi apparatus. Appreciable differences were found in the effect of these two formaldehyde mixtures: Aoyama's fixative produced fine, exuberant and complicated myelin figures in large number (fig. 4). In da Fano's, the figures were less abundant, and simpler in shape, with a tendency to assume bulky vesicular and ampular forms (fig. 3). The time found to be necessary for the appearance of myelin figures was also different: 2 to 3 hours for the first mixture, 4 to 12 hours for the other, periods which correspond closely to the respective fixation times recommended by the two procedures. Conditions for the production of myelin figures by means of Aoyama's and da Fano's mixtures were most favorable if the tissue pulp was allowed to remain on the slide, in contact with the fixative, for 10 to 20 minutes, before being crushed under a coverslip into a disc preparation. Tissues partially fixed in this manner were hard to crush, and consequently gave disc preparations which were relatively thick and opaque. However, in the most transparent parts of such preparations, numerous extracellular myelin figures could be observed (figs. 3 and 4), and in favorable

locations, definite intracellular figures could be recognized. The method previously used of crushing fresh tissue pulp, and immersing the resulting disc preparations into the fixative, was less demonstrative. As in the case of Nassonov, a similar, but simpler ring pattern caused by dissociated diffusion was noted. In such preparations myelin figures were rare or absent. The factors presumably preventing the formation of myelin figures under these conditions will be discussed later.

The experiments reported and carried out with the most commonly used Golgi apparatus fixatives indicate that myelin figures form only when a dissociated action of the fixative components can be realized. The most adequate fixative in revealing the Golgi apparatus, i.e., the Nassonov mixtures, is also the one most effective as regards intracellular myelin figure induction. Likewise, the time recommended for fixation and the time necessary for the appearance of myelin figures were found to correspond closely for a given fixative mixture. Although these facts were established in disc preparations, the experimental conditions strongly suggest that the same processes occur in tissue blocks, when immersed in the same fixatives. It is noteworthy that the topographic occurrence of myelin figures in disc preparations was found to parallel the distribution of the Golgi apparatus within a tissue block. Neither structure is found in the cells of the narrow peripheral, rapidly fixed zone, whereas both are present in the deeper layers, where the fixation is delayed and takes place by the successive action of the dissociated elements of the fixative mixture. The characteristic topography of the Golgi apparatus in tissue blocks was noted early (Sjövall, '06) and has been repeatedly confirmed (Owen and Bensley, '29; Macdougald, '35).

B. Effects of osmium impregnation. Current techniques included a thorough washing of the fixed tissue blocks, either in tap or distilled water, prior to impregnation. Accordingly, fixed tissue disc preparations were washed in the same way for 12 to 24 hours. It was found that this treatment did not affect the myelin figures already present.

For impregnation, disc preparations fixed in Nassonov's and washed were immersed in 2% aqueous OsO_4 solution, and maintained at room temperature for periods from one to 6 days. Periodic examination of the disc preparations immersed in OsO_4 solutions showed a progressive blackening of the myelin figures, including those more peripherally located in the disc, and which had already been subjected to a beginning of osmication during fixation. It was noted that prolonged stay in the impregnating solution did not affect appreciably the morphology of the myelin forms.

These observations demonstrate that myelin figures which form intracellularly during fixation become stabilized by the end of this process so that their morphology is not apparently modified during the subsequent washing and impregnation. The latter renders the figures more clearly visible, and probably

makes them more resistant to the solvent action of absolute alcohol and xylol, which are used in the last stages of the techniques for dehydration, clearing, and mounting purposes. In the present experiments, no attempts were made to use silver nitrate as an impregnating agent.

C. *Effects of acidification.* The fact that intracellular myelin figures can be induced by mixtures as different chemically as those of Nassonov, Aoyama, or da Fano suggests that their formation is not conditioned by any component of these mixtures individually, but may result from conditions such as autolysis, special pH modifications, etc., similarly involved in each procedure. Experiments at this point indicated that autolysis itself was not sufficient to account for the elaboration of intracellular myelin figures. On the other hand, all the fixatives employed in Golgi apparatus techniques are either strongly acid,[2] or else, if formaldehyde is present, have a secondary acidifying effect on the tissues (Zeiger, '30). Therefore, the influence of high H-ion concentrations on the formation of myelin figures was investigated. Rat liver homogenates were buffered at pH's from 7.7 to 3.0, kept at room temperature, and samples examined periodically, in the course of 24 to 48 hours. The observations made with such material can be summarized as follows:

Extracellular myelin figures. Myelin figures began to form in the neighborhood of pH 6.8. They were extracellular, thin-walled, exhibiting simple, vesicular or ampular forms, but of fairly large sizes. These figures were unstable and were apt to disintegrate. As a rule, they were found as sessile formations on the periphery of big cell clumps, as they were slowly migrating from blood vessels, biliary ducts, or accidental cracks leading out from the cellular mass. At lower pH's, for instance, between pH 6.4 and pH 6.0, individual myelin figures could be seen to arise at the surface of isolated cells as thin-walled, vesicular blobs. Down to pH 6.0, the extra-cellular myelin figures produced were more numerous and complex, as more acid media were used. However, at this acid range, the morphology of the intracellular lipid inclusions was not visibly affected. This would suggest that the extracellular, sessile figures and blobs were probably formed at the expense of the phospholipids of cell membranes. Below pH 6.0 the thin-walled figures became proportionally less numerous and soon failed to form, while thick-walled, highly refringent, complicated, tubular myelin figures appeared and became rapidly predominant.

Intracellular myelin figures. As already noted there were no intracellular myelin figures detectable between pH 7.0 and pH 6.0. Below pH 6.0, the lipid inclusions in the intact cells appeared first to swell into large vacuoles, then become progressively transformed into polymorphic bodies through irregular

[2] Nassonov's (potassium dichromate, 3%, 2 parts; chromic acid, 1%, 2 parts; osmium tetroxide, 2%, 1 part), pH = 1.55. Aoyama's (cadmium chloride, 1 gm; formaldehyde, 40%, 15 cm³; distilled water, 85 cm³), pH = 3.2. Da Fano's (cobalt nitrate. 1 gm; formaldehyde, 40%, 15 cm³; distilled water, 100 cm³), pH = 3.4.

budding. Below pH 5.8, definite myelin figures with particularly thick and refringent walls appeared in certain cells (figs. 5 and 6). Below pH 5.2 the incidence of intracellular, as well as extracellular myelin figures decreased rapidly and they failed to appear all together under pH 4.8 to 4.4. The time required for the appearance of myelin figures induced intracellularly by definite H-ion concentrations was shorter in saline than in sucrose homogenates. In saline they could be detected after 3 to 4 hours; in sucrose after 5 to 8 hours. They continued to grow in size and complexity for the next 24 to 48 hours, when they began to disintegrate.

Myelin figures induced by Golgi apparatus fixatives and by acidity were found to exhibit the same polymorphism, growth particularities, and typical intracellular distribution as the figures produced by ethanol and described in a previous paper. Like these, they presented the·morphological and topographical features usually considered as typical for the Golgi apparatus of liver cells. Thus, they appeared as solid or canalicular networks (fig. 1), scattered polymorphic bodies (fig. 2) or canaliculi (figs. 5 and 6), "poly-systems" and "mono-systems." They were encountered either in the perinuclear location (figs. 1 and 5), which is considered as characteristic for the apparatus during its presumed "resting phase," or in the peripheral, peribiliary disposition (figs. 2 and 6) that the organelle supposedly assumes during the "discharge phase" of its secretory cycle. Other examples illustrating the close similarity between Golgi apparatus and intracellular myelin figures have been presented in a preceding paper (Palade and Claude, '49). In that work advantage was taken of the fact that myelin figures induced by ethanol and stained with Sudan black lend themselves exceptionally well to observation and microphotography.

2. Experiments on Models

The observations so far reported indicate that intracellular myelin figures displaying the characteristic features of the Golgi apparatus can be induced, under various conditions, at the expense of lipid inclusions pre-existing in the living cells. It was of interest to find out whether myelin figures with similar features could be induced at the expense of lipid droplets of known constitution, under the experimental conditions imposed by the Golgi apparatus techniques. For this purpose, emulsions of phospholipids, triglycerides, and mixed emulsions of both were used. The tests to be described deal with the effect of basic dyes, commonly used in supravital staining, when applied on such lipid emulsions, and with the effect of Nassonov technique, i.e., fixation and osmium impregnation, on the same emulsified lipids embedded in gelatine blocks.

Staining of lipid emulsions. Lecithin emulsions in 0.15 M saline precipitated when brought in contact with 1:1,000 to 1:2,000 water or saline solutions of neutral red, methylene blue, or Nile blue sulphate. Within the reticular precipitate, which was faintly colored, there soon appeared very small spherical

bodies, 0.5 to 1 μ in diameter, brightly colored in red (fig. 7). In 30 to 45 minutes these bodies transformed into small vacuoles which continued to concentrate the dye and to increase in size (fig. 8). After one hour, typical myelin figures developed from the vacuoles (fig. 9). When stronger, i.e., 1%, dye solutions were used, the stain was rapidly removed from the medium and concentrated inside the growing myelin figures, which soon appeared as deep colored bodies on a colorless background. In contrast, the droplets of an olive oil emulsion failed to stain with neutral red or methylene blue. Seldom, faintly colored superficial membranes could be distinguished at the surface of some of the largest olive oil globules. When mixed lecithin-olive oil emulsions were used, certain characteristic forms were constantly encountered. At the beginning, a thin, continuous, faintly-colored membrane could be detected at the surface of practically every olive oil drop, indicating the formation of phospholipid membranes at the oil-water interface. Soon after, a deeply stained crescent developed, made up of membrane material which generally retracted towards one pole of the oil globules (fig. 10). Around and among agglutinated oil droplets, the stained membranes and crescents formed foam structures in which the colorless droplets appeared embedded like pebbles in pudding stone. After a certain time, the colored crescents gave rise to definite myelin figures. These transformations could be readily observed with neutral red or methylene blue, but were particularly impressive with Nile blue sulphate because of the differential staining obtained: the olive oil drops stained orange red, whereas their phospholipid membranes and crescents appeared deep blue or purple.

These staining experiments indicate that certain basic dyes can be used to differentiate phospholipids from triglycerides in mixed emulsions. At the same time they demonstrate the ability of myelin figures to concentrate basic dyes, precisely those recommended for the vital staining of vacuoles (Parat, '28), or the Golgi apparatus (Worley, '44b). These observations support the view that vacuoles and crescents are actually elementary myelin figures originating from a massive droplet in the first case, or from a membrane in the second.

Tests on embedded lipid emulsions. Lecithin emulsion in distilled water was embedded in gelatine, as previously described, and small blocks cut from the gelatine thus prepared were treated according to the Nassonov technique. From identical blocks, sections were cut before, and at various intervals during impregnation. Preparations fixed, but not subjected to impregnation, failed to show any osmicated inclusions. Even after 3 to 4 days impregnation, the only material that appeared osmicated consisted of small, 0.5 to 1 μ, hazy, brown granules scattered in the gelatine matrix (fig. 11).

Entirely different results were obtained if 0.15 M saline was used instead of distilled water for the preparation of lecithin emulsions. In such preparations, examined prior to impregnation, polymorphic bodies could be already recognized because of their slight blackening by the OsO_4 of the fixative. In sections made

at various intervals during subsequent impregnation, these bodies became more and more apparent, as a result of progressive osmication. The morphology of these inclusions was found to vary a great deal according to their location in the gelatine block. The conditioning factor in this case was the variation in local gelatine concentration produced by uneven evaporation during the setting of the block. Thus, in zones of greatest gelatine concentration, i.e., in zones exposed to desiccation during the setting of the gelatine, the inclusions were in the form of irregular, elongated bodies, their orientation in the block being determined by the dominant direction of the fibrillar network of the gel. In the regions of less gelatine concentrations, definite myelin figures were constantly encountered. The reason for these local differences was the variable degree of resistance presented by the gelatine to the expanding myelin figures during their growth. In the softest parts of the gelatine, myelin figures may reach relatively huge sizes, and subsequently collapse, the residual figures and shrunken bodies leaving around them large cavities in the gelatine (fig. 12). Between the polymorphic bodies of the dense zone, and the definite myelin figures of the soft regions, all the possible intermediate forms could be found, demonstrating, as in cellular material, the important role played by the available space for the expansion and the final appearance of a myelin figure.

Olive oil emulsions in distilled water or saline, embedded in gelatine and treated in the same manner, showed that triglyceride drops, in the present case about 0.5 to 3 μ in diameter, retained their spherical shape through all the stages of the Nassonov technique as illustrated in fig. 13. In contrast with phospholipid inclusions, the osmication of olive oil drops was rapid and intense, a deep blackening of all the oil globules being observed already at the end of fixation.

Mixed emulsions of phospholipids and triglycerides were prepared by blending 2 gm of lecithin with 1 gm of olive oil in 97 cm^3 saline. These emulsions were embedded in gelatine and the preparations thus obtained were subject to the Nassonov technique. In such preparations the triglycerides, although present in the proportion of 33% of the total lipids, did not appear as individual spherical drops. Instead, the inclusions produced by such lipid mixtures were found to exhibit the characteristic morphology of plain lecithin figures, i.e., polymorphic bodies in dense gelatine regions, and definite myelin figures, intact or collapsed, in the looser regions of the gelatine block (fig. 14). That the triglycerides had been incorporated in these structures was demonstrated by the fact that osmication was appreciably more rapid than in the case of plain lecithin figures, blackening being already advanced by the end of fixation.

These experiments on models permit the conclusion that phospholipid droplets, under the conditions of the Nassonov technique, are transformed into myelin figures during fixation, these myelin figures being subsequently blackened during impregnation in OsO$_4$ solutions. The same experiments show that

mixed phospholipid-olive oil droplets, containing as much as 33% triglycerides, can also form myelin figures without apparent residues. According to the present knowledge concerning the structure of myelin figures, this implies that triglycerides are incorporated in the walls of such figures as impurities, or that only their fatty acids, following hydrolysis, participate in the formation of the molecular films. The results show further that a definite amount of NaCl is needed to insure the formation and preservation of such figures. No myelin figures were obtained from emulsions in distilled water, whereas definite figures developed from lecithin in saline suspensions. This difference is reflected in the macro- and microscopic appearance of the emulsions. Lecithin in saline gives emulsions which are definitely opalescent, practically all the material being dispersed in the form of individual droplets varying in diameter from 0.5 to 1 μ. In distilled water, a large proportion of the lecithin has a tendency to go into colloidal solution, the size of the few remaining droplets being close to the limit of resolution of the microscope. Such emulsions are translucent.

3. Experiments with Centrifugally Isolated Cell Fragments

It has been shown previously that tissue homogenates could be fractionated by means of differential centrifugation, the cellular components present being separated according to their sizes and densities (Claude, '46; Hogeboom, Schneider, and Palade, '48). In the present work, lipid droplets liberated from the broken cells, and other lipid-containing cell components, namely, large granules, mitochondria, and microsomes, were isolated from liver homogenates. The original tissue suspension and the washing of each fraction were carried out in a variety of media, including neutral distilled water, isotonic saline, and 0.88 M sucrose solution. The effect of basic vital dyes on these isolated cell fractions in suspension and the effect of Golgi apparatus techniques on the same fractions, embedded in gelatine, were investigated. An account of these preliminary tests follows.

Lipid inclusions. The lipid layer which separated centripetally at the top of the liver homogenate consisted of small, refringent droplets, approximately 0.5 to 3 μ in diameter. When separated in distilled water or saline suspensions, these droplets failed to stain with neutral red or methylene blue; Nile blue sulphate stained them pale orange-red, while Sudan black blackened them intensely. If included in gelatine and treated according to the Nassonov technique, they retained their spherical shape unaltered, and did not form myelin figures. From these observations, therefore, it would appear that the lipid layer, separated centripetally from water or saline suspensions, consisted predominantly of neutral fat drops. On the other hand, the lipid layer separated from 0.88 M sucrose homogenates comprised, in addition to such neutral fat drops, an appreciable proportion of droplets that stained deeply with neutral red and methylene blue, and were colored blue or purple by Nile blue sulphate.

From these stained droplets the rapid development of vacuoles, crescents and definite myelin figures could be followed. The various figures so produced showed a remarkable ability of concentrating the vital basic dye employed and were entirely comparable to those which developed under similar conditions from lecithin or lecithin-olive oil emulsions. The sucrose-isolated lipid fraction, embedded in gelatine and treated according to the Nassonov technique, showed both myelin figures and unaltered neutral fat droplets. These observations indicate that the use of sucrose, because of higher density of the medium and better preservation of the material, makes it possible to separate centripetally phospholipid and mixed lipid inclusions, in addition to the neutral fat droplets obtained from water or saline homogenates.

Large granules and mitochondria. Large granules, freshly prepared in distilled water or saline, precipitated and disintegrated when treated with neutral red, methylene blue, or Nile blue sulphate. From the precipitated masses, deeply colored myelin figures were observed to develop within a few hours, their appearance and growth being somewhat slower in saline than in water. In both media, the formation of myelin figures could be considerably accelerated through partial desiccation of the stained preparations, or by treating the large granule suspensions with ethanol to a final concentration over 60%. Doubtful myelin figures were occasionally found in preparations obtained by applying the Nassonov method to gelatine blocks in which large granules had been embedded.

Mitochondria in 0.88 M sucrose, treated with basic vital dyes, were found to be considerably more resistant to disintegration than large granules in distilled water or saline. In such mitochondria preparations, myelin figures began to appear only after one to 3 days. No myelin figures were induced by ethanol, except when concentrations higher than 65% were used. No myelin figures were detected in preparations obtained by embedding sucrose suspensions of mitochondria in gelatine blocks, and treating these according to the Nassonov technique.

Microsomes. Microsomes were found to be remarkably resistant to disintegration. In the presence of basic vital dyes, 3 to 4 days elapsed before a few myelin figures appeared in preparations containing microsomes separated from distilled water or saline suspensions, and even longer with the corresponding material isolated from 0.88 M sucrose homogenates. The formation of such figures could be accelerated by treating the material with high ethanol concentrations, and especially by exposing it to desiccation. Negative results were obtained with microsomes included in gelatine and treated thereafter according to the Nassonov method.

In the preceding experiments the phospholipid-containing droplets isolated by centrifugation from 0.88 M sucrose homogenates were the only cell inclusions which gave rise to myelin figures when embedded in gelatine and sub-

jected thereafter to the Nassonov procedure, i.e., the most commonly used Golgi apparatus method. As already stated, the same droplets were found to exhibit a high affinity for neutral red, and methylene blue, under experimental conditions comparable to those of supravital staining, except for somewhat higher dye concentrations. As is known, the same basic dyes have been reported to stain vitally or supravitally inclusions said to represent the Golgi apparatus in living cells.

The phospholipid-containing droplets, still visible in freshly prepared water or saline homogenates, are subsequently destroyed, or displaced centrifugally and, therefore, included and lost in the large granule fraction. This would agree with the results of centrifugation experiments carried out with phospholipid emulsions, which showed that lecithin droplets in distilled water, and especially in various isotonic saline media, have a density greater than that of the medium.

Myelin figures were observed to develop from mitochondria and microsomes only after these had undergone disintegration as produced by autolysis, basic vital dyes, desiccation, and ethanol in high concentrations. These observations support the view that, in mitochondria and microsomes, the phospholipids known to be present are chemically bound with other constituents, especially proteins.

DISCUSSION

The work reported in this paper has demonstrated that myelin figures are induced in liver cells by certain H-ion concentrations and especially by the fixatives most commonly used in Golgi apparatus techniques. Further, it has been shown that myelin figures so produced blacken during the subsequent osmium impregnation, at the end of which they have been found to be identical in appearance to the Golgi apparatus of the respective cells, both structures exhibiting the same morphological and topographical features.

Only slight morphological differences can be noticed between the myelin figures produced in tissue discs or tissue homogenates and the Golgi apparatus as it appears in current histological sections, the latter structure being generally more distorted, and its outline less uniform. Such slight differences can be accounted for by the deformations to which the myelin figures are subjected at certain times of the Golgi apparatus procedures. For instance, fixation and, especially, dehydration through alcohols and clearing through xylols cause shrinkage and visible distortion, while sectioning may produce slight dislocations.

It should be remembered at this point that the only accepted criteria for the identification of the Golgi apparatus are: (a) its morphology and topography, and (b) its demonstration after the application of the classical fixation-impregnation techniques proposed for this purpose, the latter being considered as the most significant (Ludford, '24). The intracellular myelin figures conform

completely with these criteria. From these observations and considerations it can be concluded therefore that the Golgi apparatus of the classical cytology is a myelin figure, or a complex of myelin figures which develop intracellularly while the techniques used for its demonstration are applied. Direct observations in disc preparations and homogenates have shown that such intracellular myelin figures develop at the expense of lipid inclusions visible in vivo or in fresh material as highly refringent droplets. These observations have been confirmed by experiments on isolated cell fractions: lipid inclusions isolated under certain conditions were the only formed constituents which gave rise to myelin figures when included in gelatine and subjected to Golgi apparatus methods. Experiments with lipid emulsions of known chemical composition have indicated that the lipid droplets which develop into myelin figures, alias Golgi apparatus, have very probably a high phospholipid content.

The homologation of the Golgi apparatus with a complex of intracellular myelin figures makes it possible to understand and reconcile the conflicting observations and interpretations that have been advanced at various times regarding the nature and possible function of the apparatus. The various aspects of the problem, from the point of view of the morphology of the apparatus, its intracellular topography, its presumed physiological role, its chemical constitution, etc., will be considered in succession.

Morphology. The identification of the Golgi apparatus with a complex of intracellular myelin figures explains the different results obtained with similar techniques, and the different interpretations advanced for the structure of the apparatus. As is known, the latter was successively considered as a solid, canalicular or platework structure. The apparatus may appear as a complex of solid strands because of over-impregnation obscuring the lumen of tubular figures; or the walls of a figure may collapse and thus produce a similar massive appearance. If the cavity of a myelin figure persists and the degree of impregnation of the walls is adequate, a characteristic aspect is obtained. The presence of a cavity renders the center of the structure more transparent than the rim, the apparatus appearing as limited by a lamellar shell of blackened material. Such images led to the concept of the Golgi apparatus being a "duplex structure," composed of two different substances, one osmiophobic at the center, the other osmiophilic at the periphery. In the case of ampular, or vesicular myelin figures, the usually large cavities are preserved more readily, the resulting appearance being that of "mono-systems" or "poly-systems." In such cases, the shell of the system, known as the "Golgi Externum," or "Apparathülle" (Hirschler, '18) corresponds to the impregnated walls, while the "Golgi Internum," or "Apparatinhalt," is the visible expression of the cavity of a myelin figure.

Intracellular topography. The intracellular localization of artificially produced myelin figures depends primarily upon the location of the lipid inclusions in the

respective living cells. This location varies from cell to cell, but some general patterns can often be encountered according to cell types. In the so-called polarized cells, the lipid drops are found concentrated in a given region, known as the "Golgi zone," of the cytoplasm; from these inclusions arise localized myelin figures which, after impregnation, will appear as a polarized Golgi apparatus. In other cell types, which occasionally have been referred to as "bipolarized," "multipolarized" or "unpolarized" cells, the lipid inclusions are found less and less sytematically dispersed through the cell body; in these cases, the myelin figures derived from them appear as loose cytoplasmic networks or separate Golgi bodies irregularly scattered throughout the cytoplasm. In the particular case of the liver cells, the peripheral lipid droplets are the precursors of the Golgi bodies concentrated along the bile capillaries, while the central fat drops generally develop into more central and perinuclear bodies and networks.

The factors which determine the topography of lipid inclusions in living cells are not precisely known. In cells with powerful cytoplasmic currents, these inclusions generally accumulate on one side of the nucleus; for instance, in fibroblasts moving in tissue culture, they are found on the side of the nucleus facing the leading pole of the cell; in glandular cells, they gather on the apical side of the nucleus. In the cytoplasmic current system, such zones represent quiet regions possibly because of the shielding produced by the nuclear mass. This strongly suggests that the lipid inclusions are passively swept in these regions by intracellular currents, especially when these have a dominant direction of flow.

The intracellular position of the lipid inclusions can be modified, or even inverted experimentally by means of high-speed centrifugation. In their new position, the displaced lipid droplets will form myelin figures, as they would have done in their original location, if the cells are thereafter submitted to the appropriate techniques. Accordingly, such experiments (Beams and King, '34; Brown, '36; Hellbaum, '36; Dornfeld, '36; Guyer and Claus, '36) cannot be considered as proof of the pre-existence of the Golgi apparatus in vivo, or as marking "the end of the Golgi apparatus-artifact controversy" (Macdougald and Gatenby, '35). The high-speed centrifuging experiments prove only that the lipid droplets can be displaced within the cell and that their specific gravity is less than that of the cytoplasm. The streaming images obtained by Beams and King are myelin figures elongated along the path followed by the lipid inclusions during their displacement. Obviously such appearances do not justify the drawing of any conclusions regarding the physical state of the substance of the apparatus.

Physiological role. The hypothesis that the Golgi apparatus plays an important role in the synthesizing and secreting activity of the cells has found strong and widespread support among cytologists. This concept was based on elaborate interpretations concerned, on the one hand, with the apparent re-

lationship with secretion products, or excretory canals, and, on the other hand, with the intimate structure of the apparatus.

The haphazard growth of a myelin figure may occasionally bring it in contact with a bile capillary, secretion granules, or neutral fat drops. Likewise, developing myelin figures often penetrate between zymogen granules or mucus droplets which, subsequently, will seem embedded in the meshes of a Golgi apparatus. Such secondarily established associations were interpreted as indicating, or even as demonstrating, the active participation of the apparatus in the respective secretory processes. It is apparent that any hypothesis or theory based on such accidental associations has no valid basis. Similarly, deductions concerning the origin of the Golgi apparatus, and based on accidental connections between myelin figures, and nuclei or mitochondria, are irrelevant.

Consideration of the intimate constitution of the apparatus has led to another functional hypothesis according to which the "Golgi Externum" is capable of excreting or concentrating the substance of the "Golgi Internum." The latter was therefore considered as a "Golgi product," in the process of evolving into a secretory granule. The osmiophoby of the internum, as well as the possibility of restaining it with resorcin-fuchsin (Kopsch, Weigl) or with aniline blue (Kirkman, '37) after bleaching, was offered as evidence of its protein nature and considerable importance was attached to this, because the Golgi apparatus was supposed to produce biologically-important proteins such as apoenzymes (Hirsch, '39), or enzymes (Bowen, '29). The dyes used in the restaining experiments have doubtful specificity for proteins and, generally, it is difficult to ascertain whether they stain only the "Internum" or the whole apparatus (Kirkman and Severinghausen, '39). If, as demonstrated in this paper, the Golgi apparatus is a myelin figure developed during fixation, it is clear that the incidental concentration of certain substances within its cavities would not have physiological significance.

The migration of myelin figures, or the presence of phospholipids in secretion products, account for observations which have been interpreted as demonstrating the partial or total extrusion of the Golgi apparatus in the lumen of certain glands during cellular excretion (da Fano, '22; Brambell, '25; Bowen, '26).

The volume of the apparatus very probably depends on the amount of phospholipids present in the lipid inclusions of the respective living cells. This agrees with the fact that the refringement inclusions, stainable with neutral red, were found to be especially abundant in fresh thyroid cells from toxic goiter (Okkels, '34), a condition in which a hypertrophied apparatus has been repeatedly described (Okkels, '34; Welch and Broders, '40). As previously pointed out, the number of peripheral inclusions rich in phospholipids, found in liver cells, is greatly increased during secretory activity, and the same seems to obtain in other glandular cells. Correspondingly, these glandular cells show a most important and well-developed Golgi apparatus, if fixed during their

active period. Biochemical tests point towards an actual increase in phospholipid percentage in elements like muscle fibers (Bloor, '36 and '43), leucocytes (Boyd, '36), and corpus luteum glandular cells (Corner, '17; Weinhouse and Brewer, '42) during increased physiological activity. However such information is still incomplete and concerns the variations of phospholipids in whole tissue, not in a separate cellular fraction, such as lipid inclusions, which have been found to represent the precursor of the apparatus in living or fresh material.

Vital staining experiments and observations connected with yolk formation in mollusc embryos and larvae have been interpreted as demonstrating the existence in living cells of an active Golgi apparatus capable of synthesizing proteins and fats, and of concentrating vital dyes. This living apparatus appears in the form of separate granules, vacuoles and crescents which undergo secretory cycles and are specifically stainable in vivo with methylene blue (Worley and Worley, '43; Worley, '44a and b). It must be pointed out that a concentration of the same basic vital dyes has been obtained in small vesicular myelin figures, produced with commercial lecithin (figs. 7, 8 and 9) or with the phospholipids present in the various liver fractions separated by centrifugation. As it is well known, myelin figures are stainable by various basic dyes during their formation (Nageotte, '37), and they are able to concentrate them within their walls and in the aqueous content of their cavities (Dervichian and Magnant, '46a; Holtfreter, '48). Basic dyes in low concentrations favor the formation of such figures and increase their stability. On the other hand, crescentic images and foam structures like those presented by Worley can be readily obtained in a mixed emulsion of lecithin and olive oil, if stained with the above-mentioned vital dyes. To the "active chromophile" of Worley corresponds a simple, deeply-stained, crescent-shaped myelin figure, formed at the expense of the thick phospholipid membrane over an olive oil drop which, in this model, plays the role of the "chromophobe," (fig. 10). All such images can be considered as being elementary myelin figures produced during experiments under the influence of vital dyes. Whether similar myelin figures may exist in unstained, living cells has not been clearly demonstrated. Under such circumstances it is difficult to consider, as definitely proved by Worley, that in living cells there is a Golgi apparatus with simpler forms but with a physiological role as important as that ascribed to the classical organelle. According to Worley, this active apparatus has a complex chemical composition which includes lipids, phospholipids, proteins and nucleoproteins, one or another of these substances being dominant, depending on the phase of activity. The apparatus is integrated, in Worley's concept, into a complex metabolic cycle in which the nucleolus, the mitochondria and the microsomes are also involved ('46). Such far-reaching interpretations may appear somewhat premature in view of the scarce biochemical data now available.

Chemical constitution. As is known, phospholipid, fatty acid, and soap

molecules will give rise to myelin figures in aqueous media, under certain conditions. Other lipid or steroid substances like cholesterol and cholesterol esters, insoluble in water and not myelinizable when pure, can take part in the construction of myelin figures if mixed, in definite proportions, with phospholipids or other water-soluble substances, such as lysolecithin or soaps (Dervichian and Magnant, '46b; Dervichian and Joly, '46). Some of the substances just mentioned are present in living cells, in free state or in combination. If conditions are favorable, it can be expected that myelin figures may form at the expense of such compounds, especially phospholipids which are the most abundant myelinizable substances in cells.

In normal liver preparations it was observed that practically all the lipid droplets including those made up largely of neutral fat, could develop into myelin figures. This fact is in agreement with the observation that in successful Golgi apparatus preparations definite fat drops and fully-developed Golgi bodies are rarely encountered together in the same cells (Richardson, '34). Only in fatty liver preparations or in suspensions prepared with the liver of fasting animals, did an appreciable proportion of lipid drops fail to develop into myelin figures and retain their spherical shape, when treated with 50% ethanol. From these observations, it can be concluded that triglycerides also can become involved in the formation of myelin figures. Such a deduction is substantiated by the fact that in experiments with mixed olive oil-lecithin emulsions, all the lipid droplets were found to give rise to myelin figures without apparent residues (fig. 14). It is not known whether the triglyceride molecules are incorporated as impurities in the myelin figures, or whether their fatty acids alone, following hydrolysis, take part in the formation of molecular films.

Determination of the composition of the lipid inclusions will have to await their separation from the cells, possibly by means of differential centrifugation. Their isolation would make it possible to ascertain whether they contain nonlipid components such as ascorbic acid or phosphatases, substances which have been presumably detected in the Golgi apparatus region by biochemical tests (Bourne, '42; Emmel, '45).

The Golgi apparatus techniques and the mechanism of myelin figure formation. The influence of the medium on the formation of myelin figures is not completely understood. However, a number of factors favoring their production have been noted, among which the most efficient seem to be certain dilute acids,[3] especially N/100HCl (Leathes, '25).

The mechanism of this low pH effect is now known. It could be explained by a partial hydrolysis of the phospholipid molecules resulting in more soluble substances. The favorable action of certain ethanol concentrations could likewise

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[3] Contrariwise, strong acids with a pH below 4.0 have an inhibiting effect (Holtfreter, '48).

be explained by a similar partial hydrolysis, in addition to the fact that ethanol-water mixtures constitute a better solvent for phospholipids. In each case, the result is the mobilization of a large number of phospholipid molecules which are ready to pass into solution, or are available for molecular re-arrangements, i.e., myelin figure formation. The dissolution of phospholipid material, as well as the disintegration of already formed myelin figures, can be counteracted by the presence in the acid medium of certain electrolytes, the inhibiting action of these being proportional to their hydrophily. Accordingly, when a piece of tissue is fixed in an acid fixative, myelin figures will appear in lesser number and will disintegrate more rapidly than in the same fixative to which a certain amount of suitable electrolytes has been added. This is precisely the case for plain formaldehyde, as compared to formaldehyde mixtures containing 1% uranium nitrate, cobalt nitrate, or cadmium chloride. It can be assumed, therefore, that the effect of the various salts used in the fixative mixtures recommended for the demonstration of the Golgi apparatus is to increase the number and the stability of the acid-induced myelin figures by adjusting the solubility of phospholipids. This assumption appears valid, especially in the case of the Aoyama mixture which contains cadmium chloride, a well-known precipitant of phospholipids. The difference observed between the behavior of lecithin emulsions in water and that of the same emulsions in saline, when included in gelatine and treated according to the Nassonov procedure, could be explained by a similar reduction in the solubility of phospholipids under the influence of NaCl. The favorable action of electrolytes in myelin figure formation is conditioned by their nature and concentration. An increase in concentration progressively slows down and finally stops the development of myelin figures. A high initial concentration has an inhibiting effect. Among the common electrolytes, calcium and magnesium display the most inhibiting action (Leathes, '25; Holtfreter, '48). They apparently prevent the mobilization of phospholipid molecules and, consequently, block myelin figure formation. This effect probably accounts for the absence of classical Golgi networks and polymorphic bodies, except for very elementary ones, in cytological preparations fixed in a formal-calcium chloride mixture (Baker, '45).

As already shown, these two necessary factors, namely, acidity and stabilizing electrolytes, must not act simultaneously, but in succession to allow first the formation and subsequently the growth and preservation of intracellular myelin figures, alias Golgi apparatus. The degree of complexity of the intracellular figures is conditioned by: (a) the pH reached; (b) the length of time during which the cells are exposed to the suitable pH range and to an appropriate electrolyte concentration. The growth of myelin figures is brought to a standstill only when certain inhibiting concentration of electrolytes is reached, or when osmium tetroxide is introduced in the preparation. As already known, the osmium tetroxide will fix artificially-produced myelin figures (Nageotte, '37).

When a piece of tissue is immersed in an appropriate fixative mixture, the inward diffusion of its H-ions creates an acid gradient which progresses rapidly towards the center of the block. Under these conditions, orderly re-arrangement of the phospholipid molecules inside the cells begins in the zones of the block where the pH drops below 5.8, and is the most efficient in regions where the pH attains 5.4 to 5.2. The result of this re-arrangement, i.e., the formation of various intracellular myelin figures, is further favored by the arrival of the slower ions of the fixative mixture, bringing about an increase in number and stability of the figures. In disc preparations fixed in Nassonov's, the most numerous, typical and complicated myelin figures can be found within the yellow ring, where a certain amount of chrome anions is visibly present. As diffusion continues, the increase in electrolyte concentration up to an inhibitive level, or, in the case of Nassonov's method, the arrival of slow diffusible osmium tetroxide molecules stops the re-arrangement process of the phospholipids and thus fixes the already formed myelin figures.

A block of tissue is, therefore, subjected to two successive diffusion waves: the first (acid) starts the formation of intracellular myelin figures; the second (electrolytes) while favoring at the beginning their development, stops them when a greater electrolyte concentration is reached.

The diffusion conditions realized during the fixation of very permeable tissues, or of groups of separate cells, do not favor the formation of complicated, intracellular myelin figures because dissociation of the components of the fixative is reduced or negligible. This could account for the simple, crescentic, vacuolar, or granular forms the Golgi apparatus takes in certain invertebrate tissues, protozoa and ova.

As already mentioned, myelin figures are infrequent in disc preparations fixed in formaldehyde mixtures, although they are numerous in a piece of tissue treated by the same method. This discrepancy can be explained by different degrees of acidification. In formaldehyde fixation, the reaction of the fixative with the proteins liberates a certain amount of H-ions, so that the final pH of the preparation will depend on the quantities of proteins and formaldehyde brought into play. These quantities are larger in blocks than in the disc preparations; in the latter the acidification is probably insufficient to bring about myelin figure formation.

Besides an increase in the solubility of the phospholipids, acidification as well as ethanol treatment could favor the formation of intracellular myelin figures in another way, namely by precipitating some proteins, which, when in solution, may hamper the development of the figures, as ovalbumin in Leathes' ('25) experiments.

Golgi apparatus in tissue culture cells. Cells in tissue culture have proved to be a difficult material for the demonstration of the Golgi apparatus, especially when grown in liquid media. As shown in the present work, myelin figures

cannot develop when the constituents of the appropriate fixatives act simultaneously and rapidly upon the cellular material. This happens in the case of the thin preparations of cells cultured in liquid hanging drops, a situation which could explain the negative results obtained, when such a material has been used for the detection of the Golgi apparatus (Zweibaum and Elkner, '30). The same conditions of fixation are realized in the case of tissue cultures in plasma clot, if the clot and the explant are removed before fixation. In the thinly-extended cells which are retained on the coverslip and directly exposed to the action of the fixative, no classical Golgi apparatus has been found, either by light or electron microscopy. In this case, only discrete lipid droplets, accumulated close to the nucleus and showing an intense osmiophily, have been demonstrated (Porter, Claude and Fullam, '45; Porter, '49).

Myelin figures are built inside the cells only when dissociated diffusion and dissociated action of the compounds of the suitable fixative mixtures can be realized. This fact accounts for the successful demonstration of typical Golgi apparatus in cells cultured in plasma clots, providing that clot and explant are left in place during fixation (Zweibaum and Elkner, '30; Richardson, '34; Hill, '36; Macdougald, '37), thus slowing down and dissociating the diffusion of the fixative components. Similar results, i.e., demonstration of a Golgi apparatus, have been obtained in tissue cultures in liquid hanging drops only in the explant itself, or close to it, where the crowded cells duplicate, to some extent, the compact condition of a block of tissue. Studies on such material (Ludford, '27; Saguchi, '33; Macdougald and Gatenby, '35; Macdougald, '35) have always failed to show a Golgi apparatus in cells at the periphery, i.e., the thinnest portion of the growth zone, and have emphasized its presence only in cells close to, or well under the explant.

From these considerations it is apparent that, in tissue culture cells, the presence or absence of a Golgi apparatus is related to the quality of the fixation and not, as it has often been maintained, to the healthy or unhealthy condition of the cell.

The Golgi apparatus considered as an artifact. It has been suggested on previous occasions that the Golgi apparatus is an artifact, but the explanations put forth to account for its formation have been different from those presented and discussed in the present paper. For instance, the apparatus was supposed to result from the spreading of phospholipid materials on various interfaces produced in the cell by fixation (Walker and Allen, '27). Parat ('28) considered that the apparatus was produced by the fusion, under the action of fixatives, of vacuoles stainable in vivo with neutral red. Worley ('44b and '46) assumed that the classical aspects are produced by the deformation of pre-existing vacuolar elements. Such deformation was found to occur upon desiccation, treatment with hypertonic NaCl solutions, or with the Nassonov fixative mixture. Evidence presented by the authors last mentioned indicates that they

were dealing with elementary myelin figures, and sometimes with well-developed ones. Holtfreter ('46) suggested that the impregnated apparatus may be an artifact produced during fixation by the desolvation of hydrated bodies containing neutral fats, phospholipids and proteins. Sjövall ('06), working on spinal ganglia, arrived at the conclusion that the Golgi network was made up of a "myelinogenic" substance, and that it required previous swelling by water in order to become visible and take up impregnation.

Electron microsope observations have indicated another reason for a possible misinterpretation of images, this time related to instrumental deficiencies, not to defects in the technical preparation of the material. An agglomeration of discrete droplets, distinctly defined in the electron microscope, may appear as a coarse network under the light microscope because of lack of resolving power. This explanation is valid only for certain Golgi aspects, such as networks, provided that fixation be sufficiently good to preserve the individuality and shape of the lipid droplets. Needless to say, such conditions are exceptional and they are not realized in cytological material prepared according to the classical Golgi apparatus techniques.

SUMMARY

1. Myelin figures have been induced in cells by immersing thinly prepared discs of liver pulp in Nassonov's, Aoyama's, and da Fano's mixtures, i.e., fixatives most commonly used for the demonstration of the Golgi apparatus. These myelin figures were found to develop at the expense of lipid inclusions present in the fresh material as refringent droplets.

2. The evidence suggests that the formation of such myelin figures is caused by progressing zones of acidification within the tissue disc during fixation. This local lowering of pH results from the dissociated diffusion of the ions and molecules which compose the fixative mixtures. In support of this conclusion is the fact that myelin figures have been produced intracellularly in tissue homogenates appropriately buffered, i.e., between pH 5.8 and 5.0.

3. It is suggested that the development of myelin figures is favored, at the beginning of fixation, by low electrolyte concentrations; that the figures are stabilized later when the concentration of the electrolytes diffusing from the fixative is sufficiently increased; and that the slow diffusing osmium tetroxide molecules, if present, have a similar stabilizing, i.e., fixing, effect. Myelin figures which have been produced and stabilized during the process of fixation are blackened by OsO_4 during the subsequent impregnation.

4. Experiments with centrifugally isolated cell fractions showed that only lipid inclusions isolated in certain conditions develop into myelin figures when subjected to Golgi apparatus procedures. Experiments on models indicated that the ability of these lipid inclusions to form myelin figures and to concentrate basic dyes should be correlated with a high phospholipid content.

5. Myelin figures produced by Golgi apparatus fixatives have been compared with the Golgi apparatus demonstrated in tissue blocks according to accepted cytological techniques. Like the myelin figures induced by ethanol, they have been found to be identical, in morphology and intracellular topography, with the Golgi apparatus of corresponding cells.

6. It is concluded that the Golgi apparatus is a gross artifact, namely a myelin figure, or a complex of myelin figures, which develop in cells during fixation, blacken during silver or osmium impregnation, and are further distorted to various degrees by later handling.

7. The different functional concepts, previously elaborated in connection with the Golgi apparatus, are discussed in the light of the present findings. ·

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PLATES
EXPLANATION OF PLATES

All photomicrographs were taken on Kodak M plates, with a Bausch and Lomb research microscope and camera, using an apochromatic immersion objective (2 mm; 1.20; × 90) and compensated ocular (× 12.5), and a Kodak Wratten filter No. B-58. The photomicrographs were taken at 1100 ×, and enlarged to 1800 ×.

PLATE 1
EXPLANATION OF FIGURES

1 Disc preparation of rat liver, fixed in Nassonov's for 48 hours. Because of the thickness of the preparation, the cellular limits are hardly visible. Two large, complicated myelin figures forming a perinuclear wreath at left, and an elaborate network at right, have developed intracellularly under the influence of the fixative. The sharp contrast of the figures is due to a beginning of osmium impregnation.

2 Disc preparation of rat liver, fixed in Nassonov's for 48 hours. The picture shows a liver cell containing a number of myelin figures, located mostly at the periphery. A few small myelin figures can be seen, next to the nucleus. The peripheral figures duplicate the peribiliary disposition of the Golgi apparatus.

3 Disc preparations of rat liver, fixed in da Fano's mixture. Liver pulp was fixed in da Fano's for 15 minutes on the slide, then crushed in a disc preparation which was immersed in the same fixative for 24 hours. The micrograph shows a number of vesicular and ampular myelin figures developed in the liver pulp under the influence of the fixative.

4 Disc preparations of rat liver, fixed in Aoyama's mixture. Liver pulp was fixed in Aoyama's for 20 minutes on the slide, then crushed in a disc preparation which was immersed in the same fixative for 24 hours. A great number of myelin figures, most of them extracellular, have developed under the influence of the fixative. From a comparison of figures 3 and 4, it can be seen that the myelin figures induced by the fixative of Aoyama are much more numerous, more complex, and more slender, than those obtained in the da Fano mixture. Similarly, the Golgi apparatus is known to present a more varied morphology in tissues treated with Aoyama's fixative than in those treated with that of da Fano.

5 Induction of myelin figures by acid. (Rat liver homogenate in 0.15 M NaCl, buffered at pH 5.0; preparation kept 24 hours at room temperature; unstained.) The micrograph shows liver cells, the clear areas at their center corresponding to the position of the nuclei. Myelin figures are numerous and, in these cells, appear as perinuclear, polymorphic bodies recalling the perinuclear disposition of the Golgi apparatus in "resting" liver cells. A typical tubular myelin figure, with a visible cavity, can be seen in the lower right quadrant of the lower cell. The myelin figures are unstained and appear as light or dark bodies, depending on their position in the focal plane.

6 Induction of myelin figures by acid. (Rat liver homogenate in 0.07 M NaCl, buffered at pH 5.2; preparation kept 24 hours at room temperature; unstained.) The picture illustrates myelin figures which are located predominantly at the periphery of the liver cells. They duplicate the peribiliary disposition of the Golgi apparatus during the "discharge phase" of its cycle. Since the figures are not stained, their outline and cavities are not distinctly visible. Some tubular forms can, however, be recognized in all three cells, especially at the left corner of the upper cell (compare with the upper cell in fig. 11, Palade and Claude, '49).

G. E. PALADE AND ALBERT CLAUDE
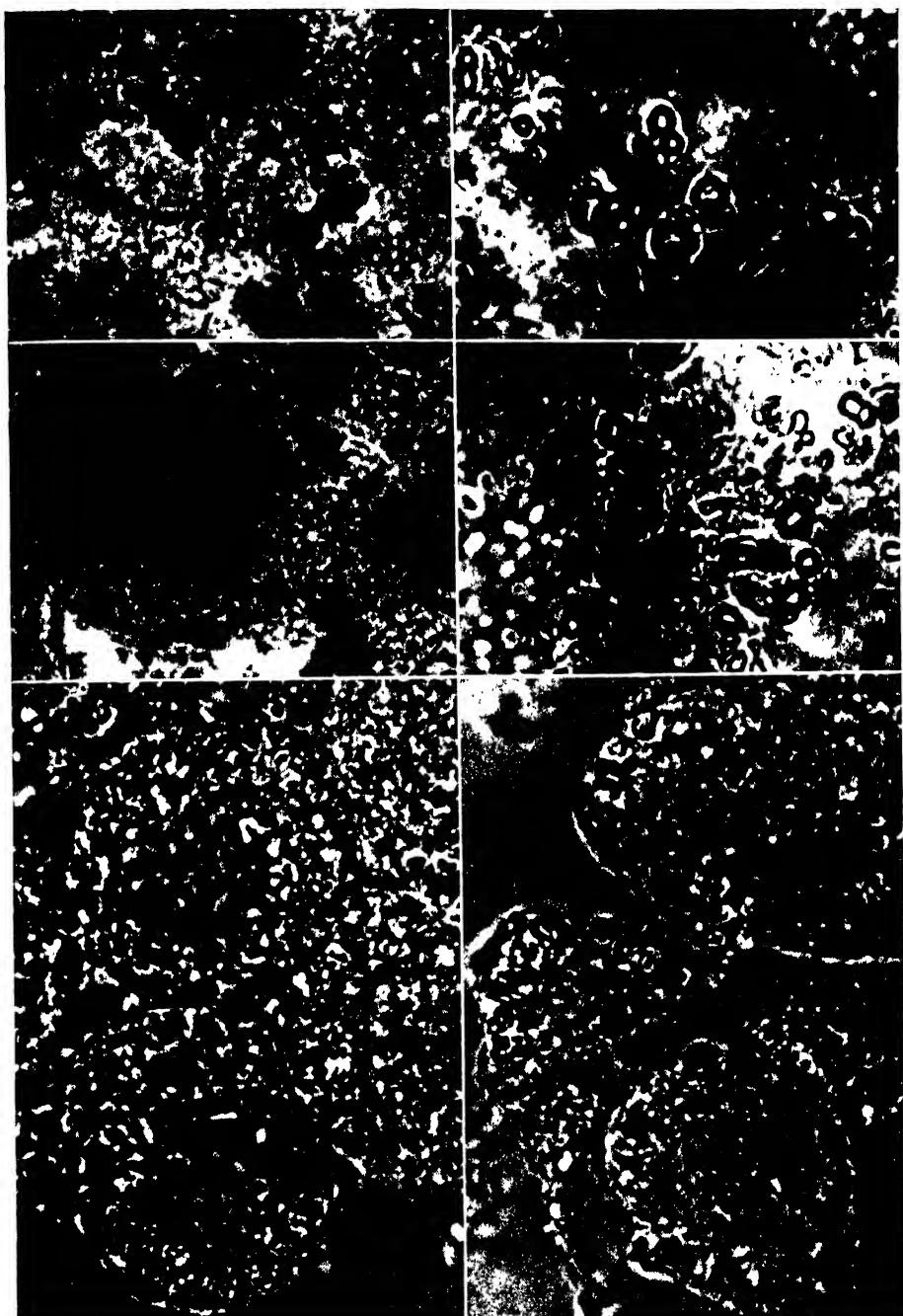
PLATE 2
EXPLANATION OF FIGURES

7, 8 and 9 Lecithin emulsion treated with neutral red. A 2% emulsion of lecithin in 0.15 M NaCl was mixed with an equal volume of 0.1% saline solution of neutral red, and the suspension examined periodically thereafter (final concentration of neutral red 1:2,000). Similar results can be obtained with methylene blue.

7 Lecithin preparation after 45 minutes contact with neutral red. The lecithin appears in the form of a reticular precipitate. Many small deeply stained vesicles have begun to appear on and out of the reticulum. The smallest are under 1 μ, the largest over 2 μ in diameter. They are elementary myelin figures intensely concentrating neutral red.

8 Lecithin after 60 minutes contact with neutral red. Many of the small vesicles have grown to a large size, while newly formed vesicles continue to appear in the precipitated lecithin mass. In general, therefore, the size of the vesicles is related to the time of their appearance.

9 Lecithin after 75 minutes contact with neutral red..Many of the orginal vesicles have evolved into definite myelin figures of various shapes, with clearly apparent cavities.

10 Mixed lecithin-olive oil emulsion treated with methylene blue. Lecithin and olive oil were emulsified together in 0.15 M NaCl (final concentration of each 2%), and the emulsion was mixed with 1:2,000 saline solution of methylene blue in the ratio 1:1. The microphotograph shows the emulsion after 60 minute contact with the dye. Some of the oil drops are coated with a phospholipids membrane and appear darker than the naked drops. Elementary myelin figures have developed at some points at the expense of these phospholipid membranes, and can be seen as deeply-stained, crescentic bodies on the side of the oil drops. These figures resemble the methylene blue stained Golgi bodies described by Worley ('43 and 44a and b).

11–14 Emulsified lipids embedded in gelatine, and treated according to the Nassonov procedure for the Golgi apparatus. Fixation: 24 hours in Nassonov's mixture; washing: 12 hours in running water; impregnation: two to three days in a 2% water solution of osmium tetroxide.

11 Two per cent lecithin in distilled water. The section shows ill-defined, small granules which are only slightly and irregularly impregnated by osmium dioxide. No myelin figures are apparent.

12 Two per cent emulsion of lecithin in saline. The picture illustrates a region at the interior of the block where the gelatine remained relatively soft. The dark bodies are partially collapsed and shrunken myelin figures, impregnated with osmium dioxide. The cavities surrounding them are produced during the expansion of the myelin figures.

13 Two per cent emulsion of olive oil in water. The picture shows spherical droplets of olive oil embedded in the gelatine, and intensely blackened by OsO_2. No myelin figures developed.

14 Mixed emulsion of 2% lecithin and 1% olive oil in saline. The micrograph shows a region at the interior of the block, where the gelatine remained relatively soft. Myelin figures, partially collapsed and shrunken, are found in cavities produced in the gelatine during the expansion of the figures. The aspect of the myelin figures is very similar to that obtained with emulsions of lecithin alone in saline.
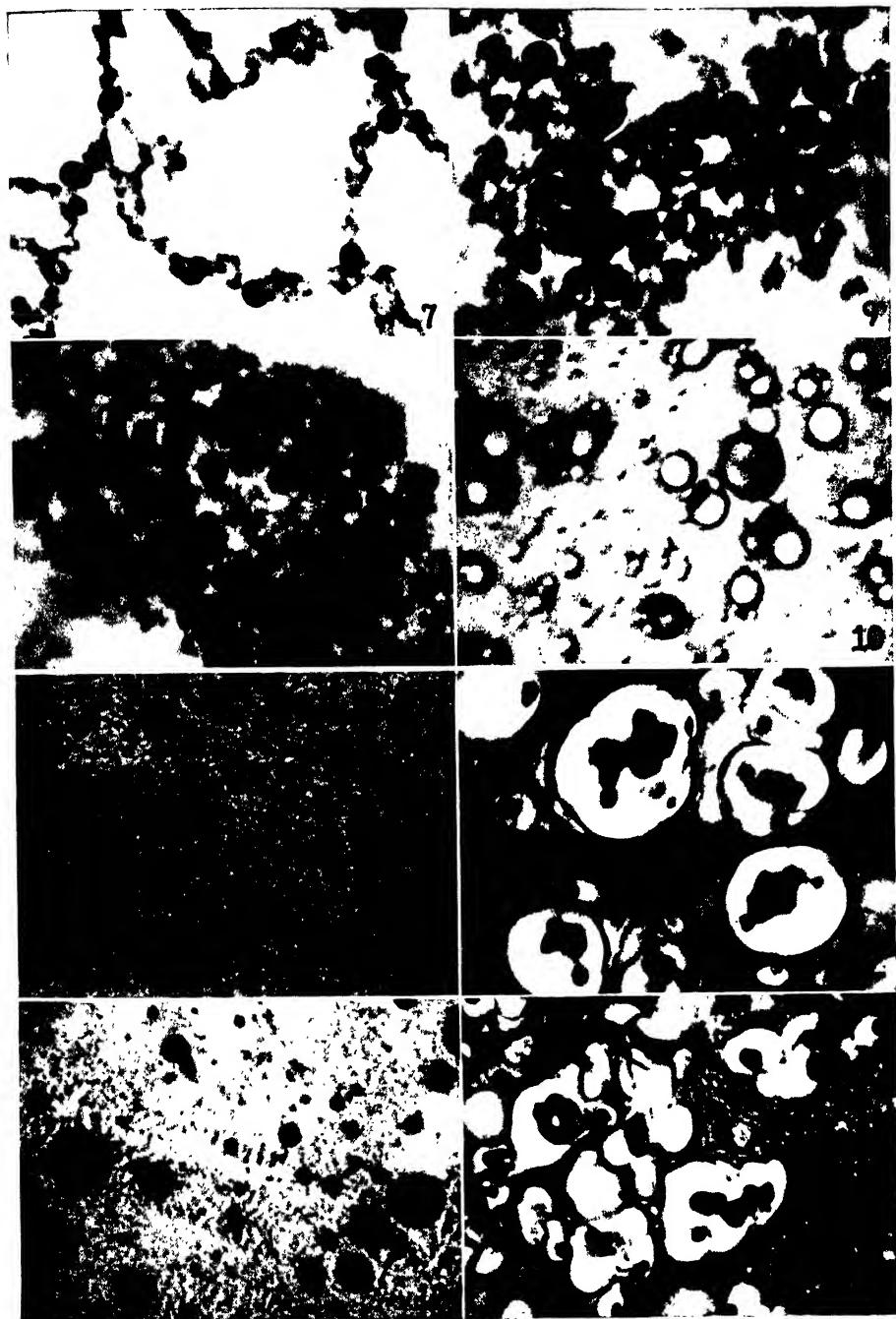
G. E. PALADE AND ALBERT CLAUDE

THE DISTRIBUTION AND STORAGE OF BLUE ANTIGENIC AZOPROTEINS IN THE TISSUES OF MICE

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(*From the Laboratories of The Rockefeller Institute for Medical Research*)

PLATES 32 TO 34

Several years ago Landsteiner and his colleagues (1–5) showed that azoproteins are antigenic, and soon after Heidelberger, with Kendall (6–8) and with Soo Hoo (9) produced a red azoprotein. Following these findings Sabin (10) and also Smetana and Johnson (11, 12) employed the product as a traceable protein for immunological and metabolic studies.

It became desirable in this laboratory to use a colored dye-protein for investigations of the physiology of the minute blood and lymphatic vessels. The linked protein of Heidelberger and Kendall did not possess sufficient tinctorial value for these purposes. Consequently, to obtain a more readily traceable protein it seemed wise to prepare a blue azoprotein from a brilliantly blue dye, in such a manner that there would be no free dye associated with it, or so little as not to produce, after injection into the body, any visible blue which could be mistaken for the azoprotein. Several blue dye–azoproteins were eventually obtained which, as a group, will be designated here by the initials AP.

The present paper describes the preparation of two of the AP and various tests made upon one of them to determine whether such a compound can be freed from unlinked dye. The fate of the dye-tagged azoproteins, following their introduction into the bodies of mice, will also be considered in relation to the general problem of the employment of tagged antigens.

Methods

To obtain the first of the AP, T-1824, a blue azo dye, was prepared in salt-free form by the method of Hartwell and Fieser (13). It was next diazotized and then coupled to the proteins of whole horse or rabbit serum, or to rabbit serum albumins, or to egg albumin. Diazotization which yielded a mono-azolinkage was considered preferable to tetrazotization and a resulting bis-azo linkage, since only half as many active groups of the protein are blocked by the former procedure and the immunological and physiological properties of the original protein should be altered as little as possible.

Preparation of Crude Azoprotein

Diazotization.—6 cc. of N/1 HCl was added to 1 millimol (960 mg.) of the dye, T-1824, dissolved in 30 cc. of water. After chilling the mixture to 10°C. a similarly cooled reagent, 1.1 cc. of N/1 sodium nitrite diluted to 4 cc. with water, was added, with stirring, and kept for 20 minutes while the temperature was maintained at approximately the same level. An

additional 0.15 cc. of the sodium nitrite in 1 cc. of chilled water was put in and the mixture allowed to stand for 10 minutes with only occasional stirring.

The excess of mineral acid was added to suppress intermolecular coupling of the dye. Under these circumstances it was not necessary to test for the acidity of the mixture since Congo red paper was always rendered blue. The sodium nitrite, too, was employed in slight excess, about 25 per cent more than the calculated amount necessary to yield mono-azo linkage. Although the excess undoubtedly caused tetrazotization of some of the dye, it helped to bring the reaction to completion more speedily and made up for the inevitable loss, by spontaneous oxidation, of some of the nitrous acid.

Coupling.—As already mentioned, the diazotized dye was sometimes coupled to whole horse or whole rabbit serum proteins and at times to rabbit serum albumin. The albumin was prepared by Howe's method (14), and completely freed from sodium sulfate by dialysis at 4°C. For coupling these proteins either 60 cc. of serum, made up to 100 cc. with water, or 100 cc. of a 5 to 6 per cent solution of albumin, was cooled to 8-10°C. in an ice bath and made alkaline with the addition of 12 cc. of similarly cooled N/1 sodium carbonate solution. The chilled, diazotized dye solution was then added, with vigorous stirring, to one or the other of these protein solutions. After standing at least 2 hours, or overnight at 8–10°C., the mixture was carefully brought to pH 7.0–7.6 with N/1 HCl, and concentrated slightly by dialysis. The resulting crude azoprotein solution contained about 3 per cent of protein and 0.7 per cent of dye. Since much uncoupled dye was present it required purification.

Purification of the Crude Dye-Azoprotein by Repeated Precipitations with Alcohol

Preliminary tests showed, in the following manner, that purification could be obtained by repeated precipitations with alcohol at the proper concentration. For example, various concentrations of alcohol were mixed with two test solutions, the first, to be called *plain dye solution*, an aqueous solution of T-1824, having the same tinctorial value as the crude azoprotein solution, and the second, to be termed the *dye-serum mixture*, consisting of 4.0 parts of whole serum and 6.0 parts of water, together with enough dye to give the final mixture the same color value as the crude azoprotein solution. When the final concentrations of alcohol ranged between 75 and 80 per cent, all dye in the plain dye solution remained dissolved, but, by contrast, the dye-serum mixture yielded a precipitate, since serum proteins are completely precipitated by alcohol in this concentration range. The precipitate was slightly colored, but when taken up quickly in water and reprecipitated the color became less. Three or four repetitions of the procedure freed practically all the dye from the protein with which it had been mixed. The final precipitate was almost white and yielded a clear colorless solution with water. In these procedures, as in all subsequent ones of a similar nature, a predetermined amount of 95 per cent alcohol was poured into the aqueous solutions containing the protein, using just enough to yield the desired final alcohol concentration. In this way the protein solution did not come into contact with higher concentrations of alcohol; the chances of denaturation were lessened, and any unlinked free dye, which would be precipitated by alcohol in concentrations higher than 80 per cent, was not brought down with the protein that was to be freed from it.

The separation of the admixed, but unlinked, dye in the crude azoprotein solution was accomplished in the same way. Successive precipitations, generally five or six, in 76 to 80 per cent alcohol, were carried out until the supernatants contained only the faintest traces of visible color. The final, almost black precipitates, when dissolved in water, gave clear, deep blue solutions.

To reduce the time of exposure of the azoprotein to alcohol, thereby avoiding as much as possible the likelihood of denaturation, almost instantaneous precipitation was brought about by the addition of 0.25 to 2.0 cc. of saturated NaCl solution. Centrifugations for only 3 min-

utes, at 4000 R.P.M., sufficed to separate the alcoholic supernatants from the precipitates, and the latter were then quickly redissolved in water. The best results were obtained when the procedure was carried out in small lots which could be handled speedily. While it was not found necessary to work with chilled reagents, nevertheless precipitation at refrigerator temperature should be less injurious to the protein, and this precaution was found absolutely necessary when the protein used for coupling was a globulin.

Next, the final precipitates were dissolved in sterile physiological salt solution, pooled, filtered through cotton, and dialyzed in the ice chest for 2 or 3 days, against water which was changed two or three times each day. When all traces of alcohol had been removed the viscose tubing containing the dialysate was hung in a stream of cold air until the contents reached the desired concentration. Dialysis was then repeated against physiological saline solution containing 2 cc. of N/1 NaOH per 4 liters. Finally the material was either filtered or centrifuged, and sealed in small tubes. To some specimens merthiolate 1: 5000 was added as a preservative.

Obviously, the material prepared and purified as described would be of little use for physiological, pathological, or immunological research if free dye remained with it which might become detached from the preparation in sufficient quantity to become visible after injection into the body. Consequently it seemed advisable to attempt to purify one of the crude azoproteins by other means and to determine the ratio of dye to protein in the resulting product. If the ratio appeared to be similar to that of the same material obtained by alcohol precipitation the fact would indicate that a relatively pure product had been yielded by both methods.

It seemed probable that chromatographic separation of the free dye from the azoprotein might be effective if a suitable solvent could be found to carry the latter through columns of paper pulp while the former became adsorbed upon them.

Purification of the Dye-Azoprotein by Chromatography

Preliminary tests were made with a variety of solvents and the two test solutions, the dye-serum mixture and the aqueous dye solution. In chromatographic columns of paper pulp, 12 per cent aqueous sodium sulfate served best since it removed all the dye from the plain dye solution and yielded almost complete separation of dye from protein in the dye-serum mixture. Accordingly this solvent was next employed to study the separation of free dye from the crude azoprotein solution. Four test solutions were employed:—a crude dye-azoprotein solution, formed by coupling T-1824 to horse serum proteins (crude T-AP-H), a mixture of the same solution with sufficient dye added to it to increase the color content by 20 per cent, the dye-serum mixture, and finally, the plain dye solution. Identical chromatographic columns 25 cm. long and 10.5 mm. in diameter, each containing 5 gm. of paper pulp, were charged with 1 cc. of one of the test solutions in 20 cc. of 12 per cent sodium sulfate solution. All the columns were developed with the sulfate solution in the usual way.

None of the free dye in the plain dye solution and only 2 per cent of the color of the dye-serum mixture came through their respective columns of pulp. On the other hand, 51 per cent of the color of the crude azoprotein solution came through and, further, the mixture of the crude azoprotein solution to which 20 per cent of free dye solution had been added yielded an interesting finding. 49.9 per cent of its color appeared in the developer, that is to say, practically the same amount of color as that yielded by the crude azoprotein solution without the addition of free dye. Clearly the pulp took out all the free dye purposely added to this solution as well as that already present in it. Since not quite all the free dye was taken out of the dye-serum mixture, calculations (here omitted for brevity) indicated that 600 to 700 gm. of pulp would be required to purify 100 cc. of the crude azoprotein solution.

Relative Purity of the Products Obtained by Chromatography and by Precipitation with Alcohol

It became a matter of interest to compare the relative purity of the dye-azoprotein prepared by chromatography with that obtained by precipitation with alcohol.

TABLE I

Milligrams of Dye and of Protein in the Crude Dye-Azoprotein Solution as Such and in Samples Passed through Each of Four Paper Pulp Columns in a Serial Chromatogram Carried out until the Dye-Protein Ratio Reached Constancy

	Dye	Protein	Mol equivalent (prosthetic groups) calculated for total serum proteins	Mol equivalent as found by alcohol precipitation method
1	2	3	4	5
Crude azoprotein solution.	6.40	30.7	21.8	
Samples	*mg.*	*mg.*		
I	3.76	23.8	15.8	
II	2.62	19.6	13.9	
III	1.90	15.2	13.0	
IV	1.23	9.7	13.2	13.0

The figures presented in columns 2 and 3 show a great loss of material. This loss is apparent rather than real since only the first portions of the effluents from each tube were used for analysis. If each sample had been collected quantitatively, after adding much highly concentrated sodium sulfate to develop the pulp column, it would have become too dilute for the Kjehldahl determination of its nitrogen.

Accordingly a sample of pure azoprotein was prepared by chromatography, as will be described, and its dye-to-protein ratio was compared with that of the product obtained by precipitation with alcohol.

Four columns of paper pulp were made in the usual manner. 1.0 cc. of the crude azoprotein solution (crude T-AP-H) made up to 20 cc. with 12 per cent sodium sulfate solution, was poured through the first column and developed with several 10 cc. batches of the same solvent. Interest was centered only in the dye-protein ratio and not in the total dye or protein content of the effluent fluids. After taking out a sample for analysis the collected fluid was poured successively through the other three columns, and a sample of the effluent from each was saved for further study.

Table I and Chart 1 give the results of the test. The first column, which contained 1.3 gm. of pulp, reduced the mol equivalent of dye in the crude azoprotein solution from 21.8 to approximately 15.8. The 3.0 gm. of pulp in the second column brought the mol equivalent down to less than 13.9, and the third column, with 2.0 gm. of pulp, reduced it to 13.0 which was unaffected by the additional 3.0 gm. of pulp in the fourth column, since a mol equivalent of 13.2 was found in this sample. The curve indicates that 6.3 gm. of pulp, or even somewhat less, was needed to remove free dye from 1.0 cc. of the solution. It is clear from the table that an approximately constant ratio of dye to protein was reached in effluents III and IV indicating that no change was to be expected on further passages through paper pulp. Column 5 shows the mol equivalent of a sample of the same crude dye-azoprotein solution purified by precipitation with alcohol.

Since the mol equivalents of the azoprotein obtained both by chromatography and by alcohol precipitation of the crude solution showed excellent agreement, although the procedures differed widely, it can be assumed that the final products were similar and relatively pure.
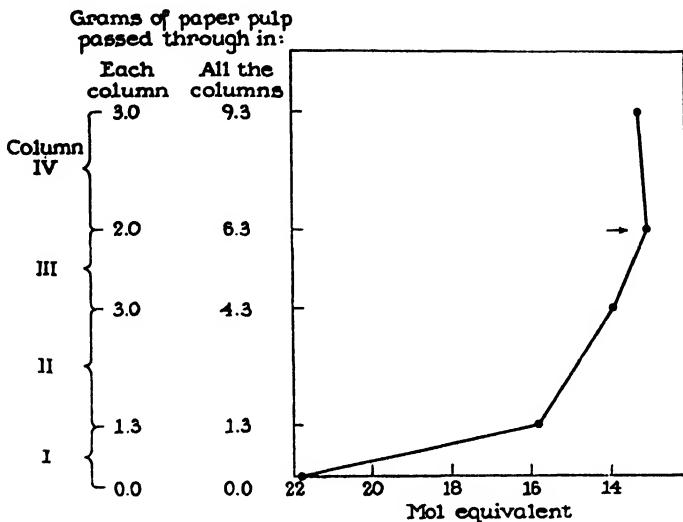

CHART 1. Grams of paper pulp required to obtain pure azoprotein from the crude azoprotein solution. The chart is plotted from the data of Table I. It makes plain the number of grams of paper pulp required to obtain, from 1 cc. of the crude dye-azoprotein solution, which contains 0.7 per cent of dye either permanently linked or free, a product having a constant molar ratio of dye to protein.

After passage through the third column (see text), the dye-protein ratio reached an equilibrium which did not change significantly in the fourth column. The arrow on the curve indicates that equilibrium was reached after passage through 6.3 gm. of paper pulp.

Difference in the Diffusion Rate of the Color of the Dye-Azoprotein and That of a Dye-Serum Mixture

Before employing the AP for physiological experiments it seemed desirable to determine whether its physicochemical behavior differed from that of a dye and serum mixture. Diffusion experiments seemed to offer a means to study the point.

In several series of tests the diffusion of a purified T-AP-H solution through 5 or 10 per cent agar was compared with that of the dye-serum mixture and the plain dye solution. The visible spread of color of the three test solutions through 10 per cent agar is shown in Chart 2 for various periods up to 98 hours. For the first 5 to 10 hours the color spread of the azoprotein and the dye-serum mixture was similar and slower than that of the plain aqueous dye solution. Thereafter, while the color spread of the azoprotein continued to be slow, the color of the dye-serum mixture began to move more rapidly and approached that of the aqueous dye solution, suggesting that, at first, the protein of the dye-serum mixture had retarded the spread of color.

This finding is in agreement with the observations of Rawson (15) and Allen and Orahovats (16) who have shown that the dye T-1824, when mixed with a solution of protein attaches itself to the latter by a cation linkage, and migrates through an electric field more slowly than the molecules of free dye in aqueous solution. The dye, in the dye-serum mixture, being in association with the protein by cation-anion linkage, will diffuse no faster than the protein itself as long as the bond is undisturbed. If, however, there is introduced into this dye-protein system, some protein hydrolysate containing anions attached to smaller, more diffusible molecules that are arranged in the same configuration as those present in the protein molecules of the dye-serum mixture,—peptone molecules, for instance—one might be able to disturb the dye-protein bond, to dissociate
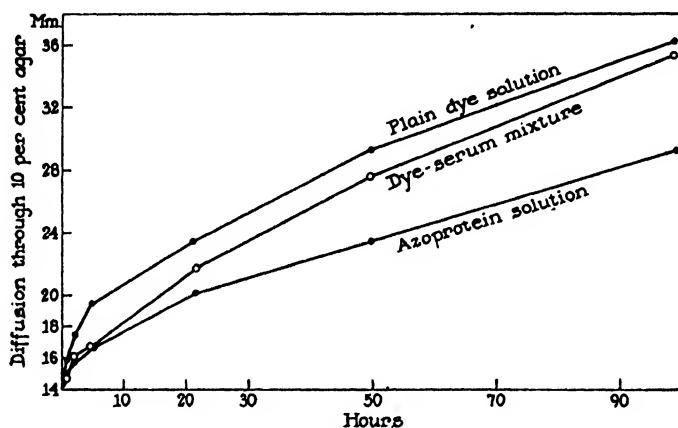

CHART 2. The rates of spread of visible color of the plain dye solution, the dye-serum mixture, and the azoprotein solution respectively through 10 per cent agar. For explanation, see text.

the dye from the protein molecules and transfer it to the faster moving protein hydrolysate. Then the rate of diffusion of color should be quite different from that of the dye that is firmly coupled to protein, as in the pure AP.

Accordingly, the preceding experiment was repeated using nutrient peptone agar. Chart 3 shows the diffusion curves obtained. By contrast with Chart 2, the color spread of the dye-serum mixture and of the plain dye, were similar in the first few hours. The movement of the former was not retarded by the protein, even initially, as in the first tests. On the other hand the diffusion rate of the color of the AP was that which one would expect from a slowly moving protein.

An AP Prepared with a Very Diffusible Dye.—For reasons that will appear below it became imperative to prepare an AP from an exceedingly diffusible dye, one so diffusible that it would escape from the body rapidly it if should

become separated from the protein to which it had been linked. The dye, echt-säure-blau B, Color Index No. 733, molecular weight 575, was found suitable, and azoproteins were formed from it using the methods outlined above. The most useful of these compounds (to be termed E-AP-G) was formed by linking the dye to bovine γ-globulin, fraction II.[1] It was readily purified by repeated isoelectric precipitation without risk of denaturation.
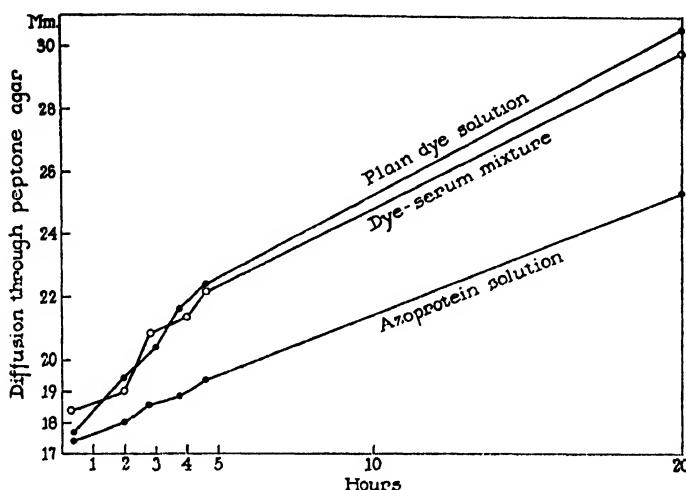

CHART 3. The rates of spread of visible color of the plain dye solution, the dye-serum mixture, and the azoprotein solution respectively through peptone agar. (See text.)

Physiological Tests

The various AP were well tolerated when given to laboratory animals, intravenously, intraperitoneally, subcutaneously, or intradermally. For example, 30 gm. mice appeared unharmed by intravenous injections of as much as 0.4 cc. of the AP solutions, containing nearly 1 gm. of azoprotein per kilo of body weight. As little as 0.05 cc. of these solutions rendered the animals blue.

It became clear in these earliest tests that the AP was taken up by the tissues in one form or another; the skin of the animals became brilliantly blue and retained its color for days. Gross and microscopic examinations of fresh and fixed tissues, prepared as described further on, showed that, after an intravenous injection of AP, blue matter appeared with great rapidity,—in as short a time as 3 to 5 minutes,—in the Kupffer cells of the liver, and after a few more minutes in reticulo-endothelial cells elsewhere in the body. More will be said of this, too, below.

The azoproteins proved to be highly antigenic, as had already been inferred they would be, from the work of Landsteiner and his colleagues (1–5). Mice

[1] Kindly supplied by Mr. Lawrence L. Lachat of Armour and Company.

injected either with the AP solutions or the proteins from which they were derived were readily thrown into anaphylactic shock by subsequent injections of the corresponding AP. The finding seemed to offer an excellent opportunity to study the whereabouts of colored antigenic material during and after anaphylactic shock and during the processes of immunization. The remainder of this paper will be devoted to a consideration of experiments planned to determine whether or not the AP can be used as tracer antigens. There follows a brief description of the localization of the antigenic material shortly after its introduction into the blood stream.

The Problem of Tracer Materials

Colored azoproteins have been used before as tracer materials for special problems (10–12, 45, 46) and so too, recently, have radioactive substances (17–19). However in studies of this sort neither the visible presence of color nor the demonstration of radioactive material in various tissues can be taken as proof that the intact antigenic material itself, as initially injected, has been localized in the tissues in which it seems to appear. Before one can assume that the whereabouts of an injected antigen has really been traced, several possibilities must be considered concerning its fate after injection.

In the case of colored azoproteins, the appearance of colored matter within certain cells of the body, even if present there only a few minutes after an injection, may indicate one of several happenings. While still in the blood the dye may be split off very rapidly from the protein, and only the former may enter the cells. The dye-protein may be taken up intact and immediately broken down, leaving only the dye within the cells. The protein portion of an AP may be partially broken down in the blood stream, allowing the cells to engulf only a modified protein, or, the protein portion may be changed intracellularly, after the AP has been engulfed.

Despite these possibilities it has seemed reasonable to believe that, since phagocytic cells can and do take up dyes (of molecular weight 500 to 900), and, at the other extreme, bacteria, India ink, and other relatively large particles, there should be no reason why these or other cells cannot take up the various AP intact, since the latter lie between these extremes having molecular weights of approximately 90,000. Accordingly, experiments were begun to learn something about the fate of AP at various periods after injection into the bodies of normal mice.

The Choice of a Suitable Azoprotein

It seemed possible to avoid some of the difficulties mentioned by a proper choice of a blue dye from which to prepare AP. Certain extremely diffusible dyes are not degraded within the organism, and form no loose or firm combinations with proteins. After injection into the blood stream of animals they do not become stored intracellu-

larly in granules, and they remain in the body only for very short periods of time. A dye of this sort, if capable of being diazotized and coupled to proteins, should form an antigenic AP that could be used to advantage to determine whether intracellular colored material found in the body after its injection into the blood stream was simply retained dye or some sort of dye-protein. Should the highly diffusible dye become split off from the protein coupled to it while it was still in the blood stream, that is to say before the AP had been engulfed by the cells, no colored granules would appear in the tissues. The dye might be taken into the cells, appear there for an hour or so as a transient coloration, and escape in the urine and bile. Should it become split off from the protein after the AP had entered the cells, its color would fade away in a few minutes as it passed out of them. By contrast the take-up of any blue material would indicate the presence of the AP since the retention of color would mean that the dye-protein linkage had not been broken, and that some sort of dye-protein, even if not the original one injected, remained within the cells.

After many trials, the blue dye, echt-säure-blau, was found to meet these requirements, and, as has been outlined above, the dye-azoprotein, E-AP-G, was obtained by coupling it with bovine γ-globulin. In a series of *in vitro* tests, this dye was added in various amounts to horse serum and to 5 per cent solutions of the γ-globulin. The dye formed no firm or loose combinations with the proteins, and the latter could always be precipitated from the dye in a colorless form.

The Behavior of the Dye, Echt-Säure-Blau, Following Intravenous Injection into Mice.— Aqueous solutions of echt-säure-blau, 0.7 per cent, containing the same amount of the dye as the E-AP-G solutions, when injected into mice in the same amounts in which it was employed later in combination with protein colored the animals visibly blue. Other mice received 2 to 5 times as much dye. All were killed, under ether or pentobarbital anesthesia, 5, 10, 15, or 30 minutes later, or at hourly intervals for 5 hours, or after 1 or 2 days. After blood specimens had been obtained by severing the great vessels in the thorax, the skin, mucous membranes, and viscera were examined *in situ* under the microscope. Fresh and frozen sections were made of liver, spleen, kidney, and mesenteric and peripheral lymph nodes, and blocks of tissue were immediately fixed in Carnoy's fluid and cleared by the Spateholz method (20).

The dye escaped rapidly from the blood, coloring the tissues diffusely blue. At no time, in any of the mice, did it appear in cells of any tissues in granular form or in vacuoles. The plasma of animals injected up to 1 hour before they were bled was brilliantly blue; fading occurred later and no blue could be seen after the 2nd hour. The bile and urine soon became intensely blue, and the skin, lymph nodes, and viscera appeared deeply and diffusely blue. However the elimination of the dye through the liver and kidneys was so rapid that all examinations made after the 3rd hour failed to disclose any blue coloration of the plasma or the tissues, either in the cells or outside of them.

The tests showed that the dye which had been present within the cells in a diffuse form never was stored as granules, and further, that it was so diffusible as to disappear from cells within 1 or 2 hours. It is clear from this that an azoprotein made from this dye should have the desired advantages outlined above.

The Behavior of a Mixture of Echt-Säure-Blau and Foreign Protein, When Injected into the Blood Stream of Mice.—Next, mice were injected with mixtures of an aqueous solution of the

dye and a 5 per cent γ-globulin solution, so made up that they contained either the same amount of dye as the E-AP-G, or three times as much. The findings were like those just described. The animals, killed at the same time intervals as in the preceding experiment, merely became diffusely blue, and then lost all color within 3 hours. No blue granules appeared in the cells at any time, and the plasma was colored for only an hour during which the bile, urine, and feces became heavily tinged. Clearly the dye did not become linked to the globulin, or to the plasma proteins, or to the cell proteins, and it was not held within the body.

Wholly different were the findings when similar tests were carried out with the echt-säure-blau azoglobulin (E-AP-G).

The Behavior of Echt-Säure-Blau Azoglobulin, E-AP-G, Following Intravenous Injection into Mice.—The E-AP-G solution, containing about 0.7 per cent of dye and 3 per cent of protein, was injected into the tail veins of scores of mice, in amounts ranging between 0.05 and 0.2 cc. per 30 gm. of body weight. Under these circumstances the amount of dye given as dye-protein was either the same or less than that used as plain dye in the preceding experiments. To study the fate of the injected AP, in the first few minutes, some mice were anesthetized with nembutal or ether and various viscera or peripheral tissues were exposed for microscopic inspection during and immediately after the injections. Individual animals were killed after 3, 5, 10, 15, 30, and 45 minutes and blood and tissue specimens were taken as will be outlined below. Other unanesthetized mice were injected with the AP and examined later, under anesthesia, at intervals ranging from a few hours to several months.

In all instances the living organs were examined under a binocular microscope, at magnifications ranging from 25 to 200 times. Next, the mice were bled from the great vessels in the thorax. Fresh contact impressions of the cut surfaces of various organs were mounted unstained, in a droplet of Locke's or Tyrode's solution under a cover slip ringed with paraffin. Some were fixed and stained with alcoholic eosin or safranin which allowed the blue azoprotein to stand out clearly, or with Giemsa stain which, of course, masked the AP. Thin, freehand sections, stained and unstained, were mounted in saline or glycerine and studied immediately. Specimens of skin from the flanks and pieces of the walls of hollow viscera removed from the body, were examined in the same way, in the fresh state and again after fixation or clearing, as will now be outlined.

Since the azoprotein is insoluble in 80 per cent alcohol and higher concentrations, tissue blocks for subsequent sectioning were immersed either in alcohol or in Carnoy's fluid. Both permanently fixed the AP *in situ*. Unstained paraffin sections, at no time brought into contact with alcohol of less than 80 per cent concentration, were dehydrated in stronger alcohols and cleared in xyol in the usual manner. Many sections were counterstained with alcoholic eosin or safranin, and others were stained in the usual way with hematoxylin and eosin. The latter procedure, necessitating the use of dilute alcohols and aqueous solutions, dissolved out AP, and sections so prepared were used only for comparison with the unstained ones.

Finally other blocks of tissue were cleared by the Spateholz method (20) after fixation in Carnoy's fluid. In these the distribution of the fixed AP could be studied to great advantage, since it retained its color well and stood out in sharp contrast to the cleared tissues.

Findings.—Following intravenous injections of E-AP-G no color appeared in either the bile or urine. In both fresh and fixed tissues, obtained only 3 to 5 minutes after an intravenous injection of E-AP-G, some blue matter could be seen, as coarse granules, or in vacuoles, in the Kupffer cells of the liver, and in reticulo-endothelial cells elsewhere in the body. The characteristics of the storage of the injected material will be described at the end of the paper, after showing that stored material was

actually the antigenic protein, for 2 days at least after its take-up by the cells of the liver. However, it is necessary for clarity to anticipate and to emphasize here that the character of the distribution and stroage of all AP preparations was similar, regardless of which dye was coupled to what protein. All produced a *Speicherung* of the reticulo-endothelial system like that which follows an intravenous injection of vital dyes of large molecule, or fine particulate matter (21–26). Tissues examined after 3 to 30 minutes, or at hourly intervals up to 5 hours showed progressively more blue, the color lying intracellularly in sharply defined granules. In animals killed after 24 hours far more blue was found within the R-E cells, the maximum take-up, after injecting the usual amount of E-AP-G—0.15 cc. of the solution per 30 gm. of body weight—occurred between the 36th and 48th hours. At this time the visible blue of the plasma disappeared. The finding is of importance in relation to the data given below.

It was of further interest that the type of cells, Kupffer cells, lymph node reticular cells, vascular endothelial cells and others, which first began to take up the colored material within 3 to 5 minutes after the injection of E-AP-G into the blood, were the same ones that continued the process during the next 36 to 48 hours. After the longer periods there were simply more of them, more intensely colored. The differences seen were only quantitative not qualitative.

The experiments show with certainty that no free dye was present in the E-AP-G employed and that no unchanged dye was split off from it in the blood stream, during the 36 to 48 hours that the plasma remained blue. Had this taken place blue would have appeared in the bile and urine. Since the preceding experiments showed that the dye itself does not become segregated within R-E cells, it seemed probable that the coupled dye-protein had been engulfed and stored.

Before this supposition could be accepted certain possibilities remained to be considered. It is conceivable that, during the process of diazotization and coupling to form E-AP-G, the dye had become changed in such a manner that it had acquired a capacity for combining with proteins. Under these circumstances, should it be split off from the γ-globulin, while in the blood, it might enter the cells and be held there. As result the material seen in the cells would be a dye-protein altogether different from the injected E-AP-G. To test the point E-AP-G was mixed with egg albumin in the concentrations in which it was found in the blood of mice following the usual injections. After standing for a while, isoelectric precipitation effected complete separation of the components of the mixture. The albumin contained no trace of color. The test indicated that the dye of the E-AP-G had not acquired a capacity for combining with other proteins and consequently, should it become split off from the globulin, while in the blood stream, it would not be held by the proteins of the cells into which it entered. As result, one can regard the blue color seen within cells, after the injections of the AP in the preceding experiments, as E-AP-G either intact or changed only in its protein portion.

Since the appearance of some of the blue material in the Kupffer cells was extremely rapid, and since it was accomplished by a phagocytic type of cell, it seemed possible that the E-AP-G had been taken up intact. The intracellular distribution of the blue matter, whatever its nature, seemed to be too rapid to allow for an appreciable digestion of the protein portion of the AP in the blood stream, since it is well known that injected antigenic proteins can be found circulating in the blood for several weeks (27–29). Nevertheless the possibility remained that the protein portion of the E-AP-G had been changed, either while still in the blood stream and before it had entered the cells, or shortly afterwards. Spectrophotometric methods failed to identify the blue material after it had entered the cells. To determine whether or not the protein portion of the E-AP-G had been changed resort was had to serological methods.

Evidence that the Blue Material Seen in the Liver Is Stored Antigen

At first it seemed improbable that one could demonstrate the presence of antigen in liver tissue since antigenic proteins injected into the blood stream remain recognizable there for weeks. As result antigen would be present in the blood within the liver, and perfusion of the organ, before attempting to extract it, would neither remove all the blood antigen nor that which might be adsorbed on the tissues.

It has just been mentioned that the concentration of E-AP-G injected into the blood stream of mice, decreases rapidly in the first 48 hours, the blood becoming colorless in that time. Consequently the antigen content of the blood circulating in the liver must also decrease, and correspondingly the amount of antigen in contact with the tissues. While this takes place the blue material accumulates progressively for 48 hours in the Kupffer cells of the liver and in R-E cells of other organs, and no blue appears in the bile, urine, or feces. If one should find that the amount of extractable antigenic material in the blood and liver followed the same pattern, that is to say that it increased in the liver while it decreased in the blood, or, if there should be, 24 to 48 hours after the injection, more antigenic material in the liver than in the blood, then one could be sure that the blue material in the cells must be antigenically intact E-AP-G itself. This would be all the more certain since the finding of blue color in Kupffer cells, following an injection of E-AP-G, indicates that the highly diffusible dye is still coupled to protein.

Accordingly tests were made to study the antigen content of the blood and livers of mice injected with E-AP-G or with bovine γ-globulin.

Preliminary studies were begun with 30 gm. mice injected intravenously with 0.1 cc. of a 5 per cent solution of the bovine γ-globulin; the same amount of protein that was used in the tests with E-AP-G. After 5, 24, and 48 hours the animals were anesthetized and bled to death. The livers were removed at once, chilled in small glass dishes standing in chopped ice, ground with sand, extracted with various volumes of 0.85 per cent saline to obtain the desired dilu-

tion, and cleared by repeated centrifugation. All manipulations were carried out in the cold. Precipitin reactions with the sera and liver extracts showed much antigen in the blood at the end of the 5th hour, and progressively less in the next 2 days. No antigen was demonstrable in the cleared liver extracts.

Since all the visible blue material in the livers of mice injected with E-AP-G was found to be intracellular it seemed probable that the γ-globulin had remained with the cloudy cellular material that was discarded in the effort to obtain clear supernatant fluids for the precipitin tests.

Obviously, then, to detect the presence of antigen in the liver, it would be necessary to include the cloudy cellular material of the liver suspensions and to resort to complement fixation tests. For the latter, the technique described by Boyd (30) was employed, freely modified to standardize it for bovine γ-globulin, as antigen, and for antiglobulin as present in the sera of rabbits immunized to the bovine γ-globulin. The system was also tested and standardized for heterologous mouse tissue. In all instances, preliminary tests were made to rule out, or to determine, the anticomplementary action of the various cells or sera used, as also that of the γ-globulin and the E-AP-G. The anticomplementary effect of the latter, in the concentrations used, was negligible. All necessary precautions (30) were scrupulously observed. In the final tests the sera and liver suspensions were diluted 10, 20, 40 times and so on by multiples of 2 up to 20,480. As in the previous tests the livers were chilled at once upon removal, ground, and extracted in the cold, to prevent enzymatic attack upon the antigen, and the dilutions were made at once with chilled reagents. Next, the mixtures were inactivated in the water bath at 56°C. for 30 minutes. It seemed better first to dilute the chilled liver suspension rapidly in cold saline and then inactivate the dilutions, rather than first to inactivate the original, concentrated liver suspension and then make the dilutions. In this way, the possibility of a breakdown of antigen by proteolytic enzymes in high concentration should be greatly diminished.

In the first tests bovine γ-globulin was injected into the blood of several mice, employing either 3 times as much protein as had been used when the E-AP-G was injected, or an equivalent amount. As result, the antigen concentration in the blood was so great that, 48 hours after the injection, and even later, the antigen contained in the hepatic blood, which is estimated (31) to amount to 20 to 40 per cent of the organ's wet weight, masked any antigen that might be present in the liver tissue, and the titers of the sera and liver suspensions were approximately equal.

Less antigen was injected in a new series of tests; 50 μg. of bovine γ-globulin in 0.1 cc. of saline. Forty-eight hours later, when the livers had been given time to take up the maximum content of blue material, as shown in previous experiments with E-AP-G, the mice were killed, and the blood and livers were taken for study. In three experiments, in each of which different amboceptor and complement preparations were used, the findings were similar; there was always more antigen in the liver suspensions. Serum readings gave in one experiment only a trace of antigen, and in the other two, 1 plus at dilutions of 20 times and faint traces at dilutions of 80 and 160 respectively. By contrast, the liver suspensions read 1 plus at 80 dilutions in the first test and 1 plus at 160 dilutions in the other two, with slight traces at 1280 and 5120 times, respectively. The end point (1 plus) was 3 tubes farther out in the liver suspension in 2 of the tests and 2 tubes farther out in the third; this in spite of the fact that the liver suspensions were greatly diluted by the liver blood which contained less antigen than the tissue itself.

Finally the tests were repeated using the blue antigen E-AP-G. The findings were similar. Indeed this was true only 24 hours after injecting 0.1 cc. of E-AP-G, which had been diluted 100 times with saline. The liver suspension yielded 1 plus at 640 dilutions, with traces at 2560, whereas the serum showed 1 plus after only 20 dilutions—a difference of 5 tubes—and traces after only 40 dilutions, a difference of 6 tubes.

Precipitin tests were also carried out in these experiments. The sera gave positive reactions at 40 to 160 dilutions in them all. In all, the liver extracts were negative; indicating, by the absence of antigenic activity in the cleared liver extracts, that the antigen, like the blue material was present within the cells and had remained with the liver detritus which was discarded before performing the precipitin tests.

The finding of maximal amounts of blue matter in Kupffer cells of mouse livers, 48 hours after injecting E-AP-G into the blood indicated, as already pointed out, that the dye-to-protein bonds had not been disrupted. The finding, in the liver suspensions by complement fixation, of antigenic activity greater than that of the blood, indicated that the engulfed blue material, seen in the cells, was still sufficiently like the material originally injected to be antigenic. It can therefore be considered as taken-up antigen.

The Azoprotein Prepared from T-1824 as a Tracer Antigen

It is to be recalled that the first of the azoproteins to be described above was made from horse serum proteins and the dye, T-1824. Although this preparation, T-AP-H, was by far the most highly colored of the AP, it could not be used for the studies just described because of the behavior of the dye in the animal body. Injections of aqueous solutions of this dye into the blood stream of mice led to the appearance of blue granules and vacuoles in phagocytic reticulo-endothelial cells all through the body, a typical *Speicherung*. It persisted for weeks. Because of this behavior the T-AP-H was open to one of the objections already discussed, namely that the dye, if split off from the protein to which it had been coupled, would appear in the R-E cells and remain there regardless of whether the split occurred while still in the blood or after the AP had entered the cells.

On the other hand, since the studies already described had shown that the dye of the E-AP-G was not split off from its protein, and further, that the AP remained antigenic in Kupffer cells for at least 2 days, it seemed worthwhile to find out whether the more highly colored T-AP-H would behave similarly. Should this be the case, the T-AP-H would serve as a much more brilliant tracer antigen than the E-AP-G and one could determine the initial sites of its distribution and early storage to great advantage. Of course, because of the ability of the dye, T-1824, to remain within cells for long periods, one could not hope to determine with it how long the T-AP-H remains as such within them, but only to trace the antigen to the sites at which it is first stored.

An experimental comparison of the behavior of the T-AP-H and E-AP-G in the bodies of mice, together with other experiments now to be described, showed that the highly colored T-AP-H serves as a better tracer than E-AP-G.

The Behavior of T-AP-H in the Tissues of Mice Compared with the Findings with E-AP-G

T-AP-H injected intravenously into mice in dosages like those employed for E-AP-G rendered the animals more deeply blue. However, the bluer plasma became colorless within 36

to 48 hours, as it did following the injections of E-AP-G. No precipitation or particle forma-
tion could be seen microscopically in the blood at any time, and although the T-AP-H colored
the plasma for many hours, no blue appeared in either the bile, urine, or feces. Blue matter
appeared in Kupffer cells and other R-E cells quite as fast as after injections of E-AP-G, the
intracellular storage of color seeming to be too fast to allow for an appreciable proteolysis of
the AP. Since it has already been shown that this does not occur with E-AP-G, it seemed
likely that it did not happen to the T-AP-H.

Fresh and fixed tissue specimens obtained, as already described, showed that the distribu-
tion and storage of the two AP were exactly alike. The similarity of behavior warranted the
supposition that the dye T-1824, like the dye, echt-säure-blau, was not split off from the
coupled protein. Further tests showed this to be the case.

Tests with Solutions Containing Not Coupled Dye.—The dyes, T-1824 and echt-säure-blau,
were added to horse serum in the same proportions as the coupled dyes of the corresponding
AP. When the resulting mixtures were injected into some mice and the corresponding AP
was injected into others, so that all received the same amounts of dye, the mice given the
dye-serum mixtures showed much color in both bile and urine, while the mice receiving the
AP solutions showed none. Further, when equivalent amounts of either of the dyes were in-
jected, in aqueous solution into the blood of mice, color appeared in both bile and urine even
more rapidly and in greater amounts than when they were introduced together with serum.

In the experiments in which the mixture of the dye T-1824 and serum was
employed most of the dye must have been loosely bound to the serum proteins
(15, 16), only very little remaining in the free state. Nevertheless the very
small amount of free dye present in the mixture was detectable through its
appearance in both the bile and urine. Since the injections of T-AP-H colored
the liver and other viscera deeply blue it is obvious that the intense coloration
could not have been produced by dye which had been split off from the protein
of the T-AP-H before it entered the cells. Had this been the case the dye, free
in the blood, would have appeared in the bile and urine. Clearly, the T-AP-H
must have entered the cells as a dye-protein. Since the dye-protein E-AP-G
retained its antigenicity for 2 days, at least, the T-AP-H chemically coupled
in the same way to similar proteins, can be supposed to behave similarly as a
tracer antigen.

The Distribution of "Tagged" Antigen in Certain Tissues ·

The azoproteins are soluble antigens. Nevertheless their distribution, follow-
ing an intravenous injection, is like that of dyes of large molecule, fine particu-
late matter, or bacteria. This sort of take-up has been so fully described (23–26),
that little needs to be said here. For the purposes of the present paper the
distribution of the colored antigens will be discussed only as implicating certain
organs known to be active either as sites of antibody formation or storage.
More will be said in later papers about the distribution of antigenic AP in
other organs and under different conditions.

The distribution of T-AP-H is exactly similar to that of E-AP-G. Both can
be readily identified in fresh and fixed specimens. Because the color of the
E-AP-G is much lighter and paler than that of T-AP-H, photographs of tissues
containing the latter have been used, the better to demonstrate the findings.

However, the following descriptions apply equally well to the distribution of both AP preparations.

Findings in Lymph Nodes Following Intradermal Injection

Earlier work from this laboratory (32, 33), since confirmed in several others (34–40), has shown that those lymph nodes which are nearest to the sites of injection of pathogenic bacteria and viruses, form antibodies in high concentration against these agents before they appear in notable amounts elsewhere in the body. Since the phenomenon was first demonstrated in the cervical nodes of mice (32), after intradermal injections of various antigens into the lymphatics of the ears, it seemed a matter of interest to study the distribution of colored antigen when introduced into these nodes by the same route and in the same manner.

By techniques already described (41–44) about 0.01 cc. of the AP was introduced into the skin of the ears of mice, under the microscope, as superficially and with as little pressure as possible. Some of the blue fluid entered lymphatics torn purposely with the injecting needle, and it slowly drained through them to the nodes. There was no visible escape of the AP from the lymphatics at any time, as happens ordinarily when plain dye solutions are injected (41, 42).

Fig. 1 a (× 25) shows an unstained paraffin section of a cervical lymph node taken from a mouse 24 hours after introducing T-AP-H into the skin of the ear. The blue antigen appears black in the photograph. It lies in the cytoplasm of the cells situated in the subcapsular, cortical, and medullary sinuses, and at the hilus. The color was not found in the nuclei although blue granules often lay so close to the latter that they were clearly outlined. By contrast the lymph follicles in the cortical portion of the node and lymphocytes elsewhere, even those immediately next cells containing the blue antigen, took up none of it. The general picture is that of a *Speicherung* with vital dyes. Fig. 1 b is a photograph of a section from the same specimen, cut at some distance from the one shown in Fig. 1 a, and stained with hematoxylin and eosin to demonstrate the lymphoid tissue. In the process of staining, while bringing the section from high concentrations of alcohol to aqueous hematoxylin solutions, the AP was dissolved out. Although these preparations are not serial sections, one can identify in Fig. 1 b many of the structures in Fig. 1 a. Comparison shows that the lightly stained areas in Fig. 1 b, that is to say the cortical and medullary sinuses and the tissue at the hilus, containing endothelial, reticular, and other phagocytic cells, are the very ones that held the blue antigenic protein.

Phenomena Following Intravenous Injection of T-AP-H

Peripheral Lymph Nodes.—Figs. 2 a and 2 b show alcohol-fixed paraffin sections, unstained and stained respectively, of a cervical node of a mouse 24 hours after the animal received the usual intravenous injection of T-AP-H (0.1 cc. of the AP solution per 30 gm. of body weight). The distribution of the antigen, all the blue material being in the cytoplasm of sinus and reticular cells, is much like that seen in Figs. 1 a and 1 b, taken from an experiment in which

the antigenic material reached the nodes directly on the lymph stream. It is probable that the node pictured in Fig. 2 also received its colored matter from the lymph after the antigen had passed into that fluid from the blood.

Mesenteric Nodes.—Following intravenous injections of the AP the great mesenteric lymph nodes invariably became deeply colored. Fig. 3 shows an unstained paraffin section of a mesenteric node removed from a mouse 24 hours after the usual intravenous injection of T-AP-H. The blue material, black in the figure, is located in the cytoplasm of cells of the cortical and medullary sinuses and at the hilus, as in the peripheral nodes; the lymph follicles and lymphocytic tissue show none of it.

The distribution of the colored matter in mesenteric nodes is better shown in transverse sections. Figs. 4 *a* and *b*, 5, 6, and 7, show, at various magnifications, cross-sections of a node from a 31 gm. mouse, 48 hours after an intravenous injection of 0.15 cc. of the blue solution. The amount of intracellular blue material, probably maximal at this time, is greater than in the previous pictures, because of the larger dosage that the animal received. Fig. 5, unstained, shows the little round nodule at the top of Figs. 4 *a* and *b*, as it appears in a different section, magnified 100 times. Many colorless lymph follicles in the cortical regions are closely surrounded by antigen-containing cells. Fig. 6, magnified 90 times shows that part of the section pictured in Fig. 4 *a*, that has been boxed in with lines. Fig. 7 ($\times$ 400) is a photograph of another section, taken from the same node. The great quantity of the antigen taken up by the cells around the sinuses is worthy of special note, as too is its absence from the lymph follicles, although many of the latter are most intimately surrounded by antigen-containing cells. The endothelial cells too contain a great quantity of the blue material. The same distribution was found in fresh imprint preparations, in free-hand sections, and in frozen ones, consequently the results cannot be attributed to artifacts produced by fixation.

Liver.—The chief findings in the liver have already been mentioned. Little needs to be added here. It should be stressed again that from the 3rd or 5th minute after injecting the AP into the blood the Kupffer cells showed the presence of the tagged antigen. As the time interval between the injection and the examination was increased all specimens, both fresh and fixed, showed a rapid increase in the number of Kupffer cells storing the material and in the intensity of color in the individual cells. The color lay in the cytoplasm of the cells not in the nuclei, and the maximal storage occurred 36 to 48 hours after the injection when the plasma lost all visible color. Between the 3rd and 24th hours the endothelial linings of some of the portal and hepatic venules often contained much dark blue, and so too did the endothelia of the sinusoids. In tissues examined after 30 hours blue was no longer visible in the endothelia. The liver parenchyma cells took no blue. More will be said of the findings in the liver in a paper dealing with anaphylactic shock as induced with the colored antigen.

Skin and Connective Tissue.—By methods described in previous papers (41, 42), the skin and cutaneous vessels in the ears of mice were observed under the microscope during intravenous injections of the various AP solutions, and at various periods thereafter. There was no visible escape of color from the normal vessels except rarely where many small capillaries entered into venules; but escape did occur from injured vessels. However after a few hours blue granules could be seen accumulating extravascularly, and sections showed that they lay in the cytoplasm of histiocytes and other connective tissue cells. The blue deposits increased in number and intensity up to the 36th to 40th hour. In contrast, when plain dye solutions of equal dye concentration were injected into the blood, visible escape of color occurred in a few minutes and the tissues became a diffuse blue. After many hours color also accumulated in connective tissue cells, but it was a more brilliant blue than that of the T-AP-H and the granulations in the cells were finer.

Other Organs.—The blue antigen was found in all organs except the brain. It was especially rich in the kidney tubules and these organs became nearly as blue as the liver and mesenteric lymph nodes. It was present in large amounts too in the muscle cells of the uteri, in the spleen the omentum, in the lungs, and in smooth muscle all through the body.

DISCUSSION

Since the AP antigens can be directly seen in fresh and fixed tissues, at high or low powers of the microscope, or in the blood, lymph, or other body fluids, if in sufficient concentration, they can be more easily employed than radioactive tracers for the study of the distribution of foreign protein that has been introduced into the body.

The findings constitute a picture of the storage of a soluble "tagged" protein antigen within the tissues of mice, following its introduction into the blood or into the skin of the ears. The sites at which the antigen is found are presumably those from which the first stimuli to antibody formation arise. It is of interest that these sites are the very ones that have been shown by classical immunology to hold particulate foreign matter, whether antigenic or not. The seizure of the colored material by reticulo-endothelial cells almost everywhere in the body indicates that the antigenic stimulus to antibody formation can be brought to bear from practically all quarters of the body upon those tissues or cells that are capable of antibody formation. There seems to be no difference in the distribution and storage of foreign material in the body, whether it is antigenic in nature or not. It is of further interest that the distribution of the AP after intraperitoneal injection was eventually the same as after intravenous injection, a matter that will be taken up in later papers.

If, then, foreign antigenic material is held within cells of the R-E system, how long does it retain its antigenicity? Is it stored and protected within the cells, or is it destroyed there even more rapidly than it disappears from the blood? These questions cannot be answered as yet, but work is going forward on the subject. Blue color has been seen in R-E cells of mice as long as $3\frac{1}{2}$ months after injecting E-AP-G. Much of the material has disappeared by that time, but, as stressed above, the fact that any color remains at all shows that some sort of dye-to-protein–linked material is still present. Clearly the breakdown of protein in the R-E cells is slow.

Mice, intravenously injected with E-AP-G in the usual amounts, show, as will be described in later work, a very rapid fall in the antigen content of the blood. By the 12th day only traces remain, but these traces continue to be present for many more days. Is it possible that the traces represent a slow output of stored antigen from the R-E cells, as the latter slowly lose their color? If so, the prolonged formation of antibody may be consequent on sustained stimulation in this way. This important matter is now under investigation.

Other authors (11, 12), working with less intensely colored dye-azoproteins, have stated their belief that azoproteins may remain intact within kidney tubule cells and various reticulo-endothelial cells for as long as a year. However, no evidence except visual has been given to substantiate the supposition.

SUMMARY

Intensely blue dye–azoproteins have been prepared by diazotization and coupling of the highly indiffusible blue dye T-1824, Evans blue, with various serum proteins and egg albumin. The products, whether purified by precipitation with alcohol or by chromatography, have a constant dye-to-protein ratio and tests have shown them to be essentially free from unlinked dye. An extremely diffusible dye, echt-säure-blau, has also been coupled to bovine γ-globulin. These materials are adapted to physiological experimentation. They seem to behave in the bodies of mice like other proteins; they fail to appear in either the bile or urine of normal animals, and they are strongly antigenic.

When these soluble antigenic azoproteins are injected into the blood stream of mice for the first time they enter reticulo-endothelial cells in almost every organ of the body; the final distribution is like that of intravenously injected, finely divided particulate matter. The azoproteins appear in the cells which classical immunological studies have shown to be active in removing particulate antigenic materials or bacteria from the blood or body fluids. The Kupffer cells of the liver and sinus and reticular cells in lymph nodes, especially the great mesenteric node, are particularly active in the removal of the blue antigens from the blood, but many other R-E cells are active to a lesser degree. The storage of the antigenic material is in the cytoplasm only; it has not been seen within nuclei, nor has it been seen within cells of the brain.

Serological methods disclose that the blue material seen within Kupffer cells of the liver after as long a period as 2 days is still antigenic in its reactions. The blue azoproteins, therefore, serve excellently as tracer antigens, especially since they can be seen directly in fresh and fixed tissue preparations and in the body fluids.

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EXPLANATION OF PLATES

PLATE 32

FIG. 1 a. A photomicrograph of an unstained section of a cervical lymph node taken from a mouse 24 hours after introducing the blue azoprotein T-AP-H into the skin of the ear. The dark areas represent the distribution of the blue antigen lying in the cells of the sinuses; the lymphocytes have none of it (see text). × 25.

FIG. 1 b shows a section from the same node stained with hematoxylin and eosin to demonstrate the lymphoid elements. Although the photographs were not taken from serial sections many of the structures in Fig. 1 a a can be identified in Fig. 1 b. In the process of staining the latter section the azoprotein was dissolved out of the tissues; consequently the clear areas, the sinuses, are those which appear dark in the unstained section in Fig. 1 a.

FIGS. 2 a and 2 b. Photographs of sections from a cervical node removed 24 hours after an intravenous injection of T-AP-H. As in Figs. 1 a and 1 b an unstained section is compared with a stained one. × 25.

FIG. 3. An unstained longitudinal section of the mesenteric lymph node of a mouse 24 hours after an intravenous injection of the azoprotein. The dark areas represent the blue antigen taken up as described in the text. × 11.

(Kruse and McMaster: Distribution and storage of blue antigenic azoproteins)

FIGS. 4 *a* and 4 *b*. Unstained and stained transverse sections of the mesenteric lymph node of a mouse 48 hours after an intravenous injection of the azoprotein (see text). × 15.

FIG. 5. The round nodule at the top of Fig. 4 *a* as it appears at higher magnification and unstained in a different section. Again the black regions represent the blue azoprotein. The lymph follicles and lymphocytes in the medullary portion of the tissue contained no color, although they were intimately surrounded by cells containing the colored antigen. × 100.

4a

4b

(Kruse and McMaster: Distribution and storage of blue antigenic azoproteins)

FIG. 6. The photograph shows the region that is included in the small rectangle drawn in Fig. 4 *a*. The blue antigen, black or dark gray in the figure, stands out in the cells of the sinuses and endothelium against the light gray of the lymphocytes. × 90.

FIG. 7. A photograph of another section from the same lymph node. This section, too, was unstained. At this magnification, a black and white print does not show the sharp contrast seen in the actual section because the lymphocytes appear gray. Nevertheless the darker appearance of endothelia and cells in the sinuses indicates the presence of the blue antigen. It seems to lie within the cytoplasm of the cells. × 400.
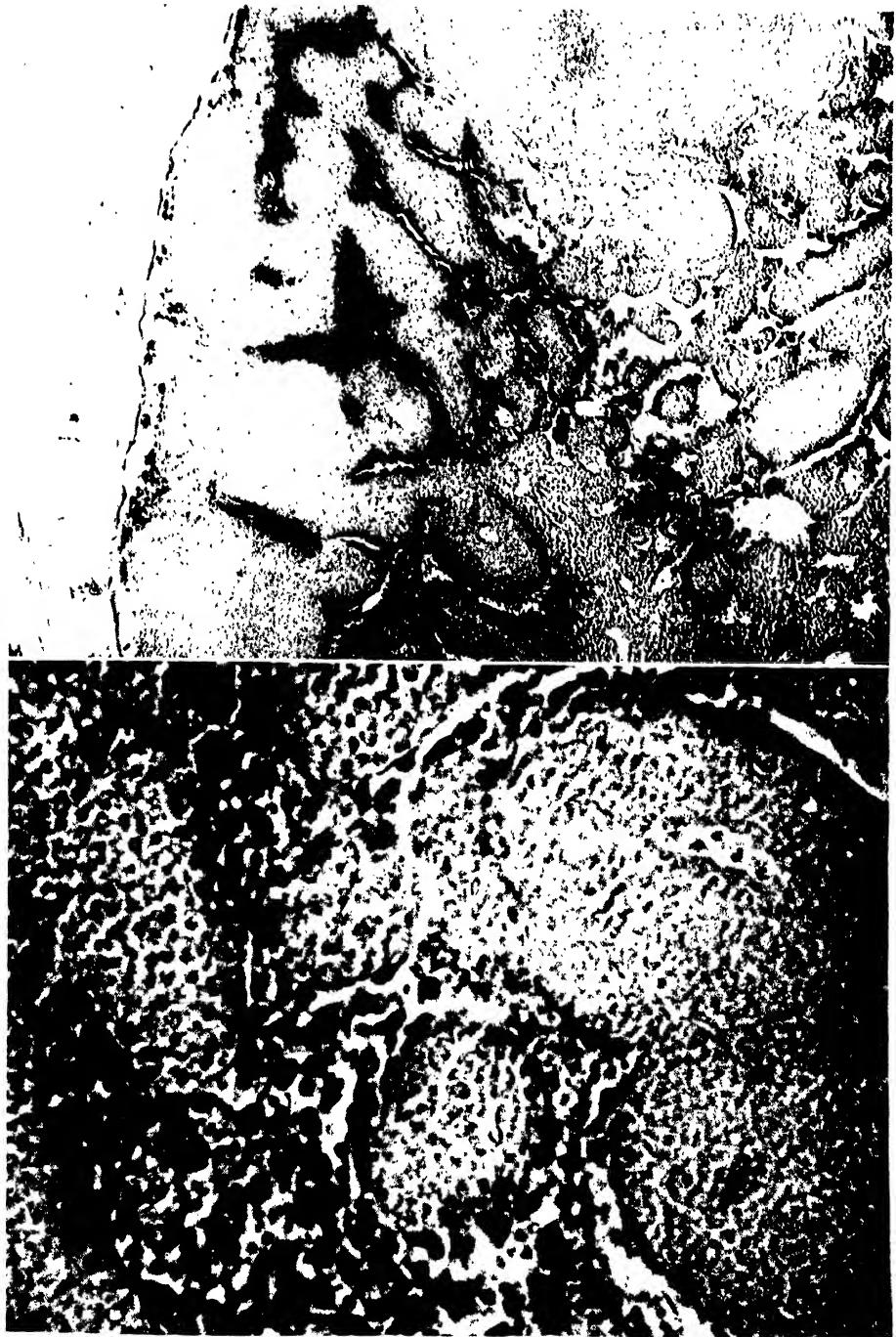
(Kruse and McMaster: Distribution and storage of blue antigenic azoproteins)

THE NEOPLASTIC POTENTIALITIES OF MOUSE EMBRYO TISSUES

V. The Tumors Elicited with Methylcholanthrene from Pulmonary Epithelium

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PLATES 6 TO 11

Benign and malignant tumors arise soon and with great regularity from the epidermis and the gastric epithelium of mouse embryos after fragments of these tissues have been implanted, together with methylcholanthrene, in the thigh muscles of adult animals of homologous strain (1–3). The present paper describes experiments, already reported in brief (2, 4), which were carried out with fetal lung. Methylcholanthrene induces remarkable changes in the transplanted pulmonary tissues, and tumors in wide variety quickly derive from it.

Method and Materials

The technique was essentially that already described (1, 3). For most of the tests mice of C strain have been used as previously, though some tests have been made with Webster-Swiss animals and those of the A breed. Embryos 18 days along, as determined by the vaginal plug method, were used in the main; they averaged 18 mm. in length. The youngest embryos employed were 15 days old (13 mm. long). The pulmonary tissue of smaller ones is difficult to procure in the necessary quantity without inclusion of the cells of other organs.

To expose the lungs, an apron-shaped flap in the body wall, extending down and across the abdomen, with its base on the upper thorax, was freed with the cautery, reflected over the neck, and pinned down. The thoracic viscera were torn out in a mass with fine curved forceps, washed in Tyrode's solution, and under a binocular dissecting microscope at × 17, the lungs were cut away and washed again. After those of the entire litter had been assembled in a little of the salt solution, they were chopped into small fragments and the resulting small quantities of tissue suspension were implanted through skin slits into the posterior thigh muscles of young adult C males. Olive oil saturated with Scharlach R and containing 1 per cent of methylcholanthrene (*OSSM*) was ordinarily introduced with the tissue, but many control implantations were made with Locke's solution or Tyrode's solution only, or else with olive oil saturated with Scharlach R (*OSS*). Just prior to use the oil preparations were shaken in a mechanical shaker with one-third as much Tyrode's or Locke's solution to make an emulsion, and this emulsion was maintained throughout the period of the injections by drawing it in and out of a syringe through a needle. Each implantation consisted of 0.025 cc. tissue suspension—containing often but a few fragments of tissue—followed by 0.075 cc. salt solution, and 0.025 cc. of oil. The oil was drawn first in the injecting syringe, then the salt solution, and lastly the tissue suspension, the interposed column of Tyrode preventing the fragments from becoming covered with oil and these latter blocking escape of the oil back along the needle track. More than 200 implantations were carried out, in 13 experiments,

each with the pooled lungs from a separate litter of embryos. The injected thighs were palpated for nodules 3 weeks after implantation and any found were drawn to size on charts, as they were also at each of the frequent later examinations prior to sacrifice. Autopsy findings were usually sketched as well as recorded, and blocks were taken in acid Zenker solution for sectioning and staining with eosin and methylene blue. Many were cut serially. When tumors were to be propagated, the transplantations were carried out with trocars, through skin slits, into the thigh muscles of young male adults of the same strain. As in the case of epidermis and stomach, special care was taken when the tumors were transplanted to exclude the possibility that any methylcholanthrene was transferred with them. Scharlach R remains even longer in olive oil than the carcinogen, as ultraviolet light has shown, and the neoplastic tissue utilized was regularly devoid of any pink coloration with the dye.

EXPERIMENTAL

Findings in the Control Implants in C Mice

The lung tissue of embryo mice of the C strain, in the latter half of gestation, regularly established itself after intramuscular implantation in Locke's or Tyrode's solution, and some of it was still living in animals kept 143 days, the longest period of observation. Occasionally the bronchiolar tissue outlasted the alveolar.

Within a few days after implantation the fragments coalesced into a grayish, translucent layer 1 to 3 mm. across, sometimes flattened to a skim by the pressure of the muscle, but in occasional instances as much as 1.5 mm. thick. It could not ordinarily be felt between the fingers. Not infrequently it was stippled with red or brown, owing to fresh or old blood pigment, or a blood-filled cyst was found in its place. There was no reactive proliferation or accumulation of small round cells about the implants, which were still doing well even after 143 days. As a rule they were separated from the muscle fibers by a few fibroblasts only (Fig. 1).

The pulmonary tissue, as obtained from near-term embryos (20 mm. long) is exceedingly cellular, its alveoli lined with cuboidal elements, and its smaller bronchi and bronchioles little differentiated (Fig. 2). Within a few days after implantation in salt solution the fragments lying next one another united to form a mass in which they could no longer be discerned individually, and maturation and differentiation went on so rapidly that within 3 weeks characteristic bronchi had formed, some with cartilage about them; the alveoli had become moderately distended with fluid, presumably of bronchial origin, and the lining cells had undergone flattening,—though this and the distention were less considerable than in the functioning lung. Often the alveoli were compressed, and there the epithelium was cuboidal, as in the atelectatic lung of the adult. Large alveolar macrocytes were frequently present ("heart failure cells") and these cells sometimes became numerous and were heavily engorged with erythrocytes or brown pigment. A few of the grafts disappeared eventually, owing, it would seem, to destructive hemorrhage into them. No tumors arose from any of the implants in salt solution, nor were any structures suggestive of them encountered microscopically.[1]

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[1] Weddell (*Arch Path.*, 1949, 48, 227) has recently transplanted fragments of mouse embryo lungs to the subcutaneous tissue of adults. "Cyst formation, bronchiectasis and bizarre epithelial overgrowths" occurred frequently.

There was good reason to suppose that the lining cells of the bronchi and bronchioles of ung fragments might respond to Scharlach R in olive oil by proliferating and becoming metaplastic; for the dye stimulates and attracts the cells of adult (5) and embryo (1) epidermis, causing them to take on a carcinomatous appearance, and it has a similar though less marked effect on the epithelium of the squamous portion of the embryo stomach (3). Yet only occasionally did it induce noteworthy changes in the lung tissue though the identical dye specimen (Grübler) was employed. These changes took the form of a mild metaplasia of the lining of the bronchi and bronchioles, where they had been cut across and a droplet lay next them, the columnar epithelium altering within a few weeks to a transitional layer and then to one stratified and squamous. The changes were never widespread nor accompanied by active proliferation, and the state of the alveolar tissues differed not at all from that when it had been introduced together with Locke's or Tyrode's solution. However in one implantation nodule with *OSS*, (out of 37 examined microscopically in serial sections), a characteristic pulmonary adenoma was found. The implant had been in place 64 days, as had another of the same tissue with *OSSM* in the opposite thigh of the same animal. This latter also contained an adenoma.

The Early Changes Due to Methylcholanthrene

Very different were the findings when methylcholanthrene was present in the olive oil in addition to Scharlach R. The pulmonary tissue proliferated actively and had usually formed a firm nodule within 3 weeks, of sausage or football shape as a rule, and 3 to 5 mm. or even 8 mm. long, but rarely more than 2 mm. thick.

Alveoli formed as usual, but the *OSSM* did not attract the lining cells as it had the epithelium of stomach and skin, none extending out to surround adjacent oil droplets. Many of the latter underwent inclusion within the pulmonary tissue as the implanted fragments coalesced, but others remained lying free, and in consequence sarcomas were a relatively frequent complication though fortunately a late one.

The epithelium of the bronchi and bronchioles was strongly attracted to the *OSSM*. Wherever the cut-across lumen of these structures lay near oil droplets the lining cells extended out in tongues and surrounded then, even when they were large, just as embryo epidermis does under similar circumstances (1). Striking metaplastic changes took place concurrently. The columnar epithelium of bronchi and bronchioles built up into a pseudostratified, transitional layer which soon became stratified and squamous, the cells shedding off at first but later forming keratinized lamellae. No squamous epithelium exists in the normal lung of the mouse.

These changes could usually be seen within 14 days after the implantation. Already a thick, keratinizing, squamous layer was covering the surface of the neighboring large oil droplets; and tongues of epithelial cells were extending to those a little further off. By the 21st day the picture was more complicated. Occasionally the epithelium of a bronchus had undergone a redundant proliferation, forming a papillomatous intrusion into its lumen (Fig. 12). More often the bronchial epithelium had a carcinomatous aspect and had actively invaded the alveolar tissue, filling its spaces and sometimes replacing it *in toto* (Fig. 3), though in other instances leaving its pattern intact (Fig. 5). Invasion occurred especially where the pulmonary tissue contained many scattered, minute oil droplets. Sometimes reactive connective tissue had formed about these latter, and the cells invaded this also.

The changes simulated cancer but after transplantation of the apparently malignant tissue to other mice, in tests made on the 51st, 64th, and 77th days, respectively, it failed to give rise to tumors (Fig. 4). The sequence of morphological events nearly resembled that reported by Passey as taking place in rats with bronchiectasis due to bacterial infection (6), but the epithelium proliferated far more profusely, gave rise to more diversified pictures, was often invasive, and in many instances extended into and largely replaced the alveolar tissue.

The changes in the alveolar elements while less dramatic were considerable. Alveoli formed as usual but the lining cells exposed to the methylcholanthrene remained cuboidal instead of flattening; there was much desquamation; and frequently after some weeks the cells situated at the periphery of the mass became close packed, increased considerably in size, and occasionally pushed out a little way into the reactive connective tissue which by now had formed about the mass. Yet it seemed unlikely that they had become neoplastic since the changes were not focal but affected the cells all along the periphery of the nodule.

A singular phenomenon was squamous keratinization of the alveolar epithelium. Sometimes an entire alveolus became filled with stratified lamellae as result of differentiation of the cells lining one side (Fig. 5). On this side the living cells usually heaped up two or three deep prior to keratinization and occasionally they formed tufts. Serial sections showed that the affected alveoli often were situated at a distance from any bronchioles, the stratified squamous layer having no direct connection with bronchial epithelium.

The proliferative and metaplastic activities of the bronchial epithelium continued during later weeks, though at a diminishing pace. In not a few instances the adjacent alveolar tissue became compressed or was largely replaced by the accumulating mass of living or keratinized metaplastic elements. The histological picture was rendered still more various as time went on by neoplastic changes superimposed on the metaplastic.

The Neoplastic Changes

It was found that the complications introduced by metaplasia could be largely avoided if the tissue for implantation were taken from along the wedged-shaped edges of the pulmonary lobes, from regions that is to say where only small twigs of the bronchial tree existed. The alveoli of the resulting nodules were then much less frequently encroached upon by the proliferating bronchiolar epithelium. Yet metaplasia of this latter was so frequent that it was still impossible to tell whether metaplasia had not preceded carcinomatosis or when this latter had superseded it. Tumors of another type arose though, which the bronchial metaplasia did not simulate, namely pulmonary adenomas. These appeared early (Figs. 7 and 8), were frequently multiple, and sometimes numerous (Fig. 9). Serial sections disclosed eighteen discrete adenomas in a single small nodule measuring $3 \times 2 \times 1.5$ mm. that derived from an implantation made 28 days previously. The lungs of the adult hosts, in contrast, very seldom showed the growths, and then they were nearly always solitary.

The adenomas which develop in adult mice as result of the action of carcinogens are similar in morphology to those occurring spontaneously (7) and have been many times described. Grady and Stewart (8) have studied the early stages of those due to methylcholanthrene. The injection of urethane into C strain animals in late pregnancy is followed by such prompt development of adenomas in the lungs of the young they carry that characteristic growths are sometimes present within 3 days after their birth and are frequent and much bigger after 10 days (9).

The earliest stages of adenoma formation in the implants of the present experiments could not be perceived because of atelectasis and because the lining of the alveoli exposed to *OSSM* tended to remain cuboidal; but the growths proliferated so actively as soon to form sizable spheres amidst the alveolar tissue (Figs. 7 to 9), becoming recognizable in not a few instances toward the end of the 3rd week. For some while the cells composing the spheres were markedly basophilic, with round vesicular nuclei and scanty cytoplasm, and lay close packed, undergoing almost no differentiation, in which respects they resembled many of the adenomas encountered in the suckling offspring of urethanized mothers (9). But as time went on they formed the characteristic acini lined with cuboidal elements, and then by all morphological criteria they resembled the adenomas of adults. Occasionally one of them was situated next a terminal bronchiole, but the large majority were wholly surrounded by alveolar tissue.

As time went on, the growths increased in number and enlarged, compressing the pulmonary tissue, and replacing it more or less completely (Fig. 9), though individually they were never more than a few millimeters across. Several that were successfully transplanted merely established themselves (Fig. 6) as did the non-neoplastic pulmonary tissue which in some instances accompanied them. But one did more. It slowly yet steadily proliferated, retaining the adenomatous morphology; and, though almost non-invasive, within 4 to 6 months formed masses up to 50 mm. across (Fig. 10). This tumor has been carried through 6 successive groups of new hosts thus far. Mitoses have been very rarely seen. In growths of the second and subsequent "generations" spicules of bone were often laid down amidst the epithelium (Fig. 11). The deposition of bone was in some instances so extensive that the whole tumor mass had a rock-like hardness. This phenomenon has already been described in adenomas transplanted from adult mice (10).

A sudden enlargement of an implantation nodule during the first 3 months, with progressive growth thereafter, generally meant that a carcinoma had arisen, sarcomas developing later as a rule. On incision of the nodule soon after this change was first noted there was usually found a solitary cyst, full of watery fluid, in which were ruddy droplets of *OSSM*, and some lumps of

TABLE I

Outcome of the Transplantations

Growth No.	Embryo length	Tumor		Character of growths transplanted	No. of hosts	Total implants	Fate of implants				Character of the Transplanted tumors
		First noted	Transplanted				Grew	Regressed	Survived	Failed	
		after									
	mm.	*days*	*days*								
1	15–17	37	51	5 mm. nodule: *squam. metaplasia or squam. carc? Adenoma*	4	4			1	3	The one successful transplant was wholly *adenoma*
2	18–20	59	64	6 mm. nodule: *squam. metaplasia, adenoma*	4	8				8	
3	18–20	77	77	12 mm. mass: *squam. metaplasia, adenomas, sarcoma*	4	8	6			2	*Sarcomas*
4	18–20	77	77	·6 mm. nodule: *squam. carc.? Adenomas*	3	6	2	3	1		*Adenomas. Sarcomas*
5	15–17	70	81	12 mm. thick-walled cyst: *squam. carc.*	3	4	1	1		2	The successful transplant was a *squam. carc.* which grew with great vigor and regularity in a 2nd and 3rd generation of new hosts
				Three discrete 1 mm. nodules, *adenomas*	2	2				2	*Adenomas*, 1 mm. across
6	18–20	85	91	5 mm. cyst: *squam. metaplasia or squam. carc.?*	4	8				8	
7	15–17	70	93	10 mm. solid mass: *3 transitional cell carcs.*	4	4	4				*Transitional cell carcs.* transferred through 3 generations of new hosts
8	17–19		102	5 mm. cyst: *squam. carc.*	3	3	1				Slow growing *malignant papilloma*
9	17–19	102	102	8 mm. cyst: *anaplastic carc. and 2 squam. carcs.*	4	4	1			3	Slow growing *anaplastic carcinoma*
10	13	94	108	10 mm. cyst: *transitional cell carc.*	4	4	4				*Transitional cell carc.* transferred through 3 generations of new hosts
11	15–17	115	123	10 mm. cyst: *alveolar cell carc.*	4	4	3			1	*Carc.* like original. Transferred through 2 generations of new hosts
12	20–23	139	188	5 mm. solid mass: *adenomas*	3	3	3				*Adenomas*, which became huge. Transferred through 6 generations of new hosts

Squam. = squamous.   (Italics indicate microscopic diagnoses.)
Carc. = carcinoma.

keratin or pultaceous material stained pink with Scharlach R. The wall of the cyst was not infrequently several millimeters thick and its inner surface rugose, but more often it was thin and one or more discrete cauliflower growths or rugose discs protruded inwards. These were the cancers; often they were multiple. As time passed the neoplastic tissue extended into the adjacent muscle though the mass continued to be cystic. When the animal was let live the tumors became huge, surrounding the femur, extending below the knee, and up over the pelvis, and frequently rupturing through the skin with liberation of much pultaceous, pink or creamy material. Eventually they proved fatal. The course of events was in other words identical in the gross with that of the carcinomas originating from the epidermis or the squamous portion of the stomach of mouse embryos (1, 3). From these latter tissues benign papillomas arose occasionally, but no such tumors took origin from the implants of pulmonary tissue.

Most of the animals were killed early, for the better study of the tumors in relation to the tissue from which they had arisen. Transplantation was frequently carried out to test whether the growths were autonomous neoplasms. Results of the transplantations are summarized in Table I. Growths described in the text can be identified in the table by reference to the day on which transplantation was carried out.

The carcinomas, though diverse in morphology, had no peculiarities suggestive of a derivation from embryo tissue. Many were papillomatous (Figs. 13 and 14), and not a few were composed of transitional epithelium (Figs. 15 to 18). A single tumor was obtained (Fig. 19) which had the aspect that in the case of human growths is usually held to betoken an origin from alveolar epithelium (11). The generality of the growths were manifestly of bronchial origin and most were of squamous cell type, though differing considerably in detail. Some bore a close resemblance to the tumors of this sort which arise from implants of the gastric lining of embryos in response to methylcholanthrene (3). Usually their cells keratinized, but in not a few cases desquamation took place before this could happen. The cancers were frequently multiple, indeed sometimes a potpourri of malignant entities, and transplantation occasionally yielded neoplasms quite different from what microscopic examination of a slice from the original growth had led one to expect. With continued transplantation, one or another of the neoplastic components eventually came to predominate as has been the general experience with other mixed tumors. For example, three widely differing neoplasms were present in the first and second generations of the tumor shown in Fig. 15, but in the third generation there remained but one, a transitional cell carcinoma (Figs. 17 and 18). In the earlier passage a singular intracanalicular growth had been prominent, com-

posed of thick connective tissue septa covered with a shallow layer of neo-plastic epithelium which was desquamating into irregular spaces (Fig. 16).

After 70 days spindle-cell sarcomas occasionally arose and after 90 days they were frequent. Serial sections of the small sarcomas showed in their midst depots of *OSSM* that had escaped encystment by epithelial cells. The results of a typical experiment will be described.

The lungs of ten embryos 15 to 17 mm. long (about 16 days old) were chopped fine in Locke's solution, and 0.025 cc. of the resulting suspension was implanted, together with *OSSM*, in one thigh of twelve adults and, as control, with the same quantity of Locke's solution but without *OSSM* in the other thigh.

The first mouse was killed after 51 days. No trace was found of the implant with Locke's solution, but that introduced with *OSSM* had given rise to a nodule 5 mm. long which was sectioned serially. Fig. 4 shows it in cross-section. It consisted of a central cyst partly lined with stratified squamous epithelium with scattered large and small islands of alveolar tissue about it. The cyst had contained ruddy oil at autopsy. It opened directly into a large bronchus ramifying amidst pulmonary parenchyma, and there was more oil amidst this latter, as evidenced in the section by rounded lacunae. Two characteristic pulmonary adenomas were present (arrows) amidst the alveoli. At the other end of the oblong nodule was what appears to be an active squamous cell carcinoma. Pieces were transplanted to the thigh muscles of four young adults, which were killed 91 days later. Only one had any nodule then, and it was only 1 mm. across. Serial sections showed it to be adenomatous in character (Fig. 6), the presumptive carcinoma having failed to survive.

A second mouse was killed 57 days after implantation. Again no growth was found in the leg where the tissue had been implanted with Locke's solution; but where it had been put with *OSSM* there was a 3 mm. mass in which 8 discrete adenomatous nodules were found in serial sections amidst hemorrhagic alveolar tissue (Fig. 9).

Mouse 3 was killed after 71 days. Where the control implant with Locke's solution had been placed there was a layer 3 mm. long and 1 mm. deep of grayish tissue speckled with brown spots. It consisted of distended pulmonary alveoli containing many macrophages laden with brown pigment. There was nothing to suggest adenomatous growth. In the thigh injected with *OSSM*, only a trace of lung tissue had survived and sarcomatosis had begun.

The next mouse, No. 4, was killed after 81 days. Where the lung tissue in Locke's solution had been implanted there was a shallow, gray layer, brown-speckled, of about the size of that found in mouse 3 and of similar composition (Fig. 1). In the opposite thigh amidst the muscle there was a cystic mass measuring 12 by 7 by 5 mm. The cyst contained pink, semisolid, necrotic material and its wall was 2 to 3 mm. thick. Pieces of the wall were implanted in three young adult mice, and the rest taken for section. The microscope showed that the cyst had contained jumbled masses of keratin, and its wall was lined with what appeared to be a squamous cell carcinoma of papillomatous tendency (Fig. 13). Transplantation proved that a cancer was indeed present, for the graft succeeded in one of three new hosts and the result-ing tumor was passed to a second and a third group of mice in turn before it was discarded, the carcinoma growing in all three hosts of the third generation. It was still cystic but the cysts were now lined with almost filiform papillomatous protrusions covered with living, keratinizing epithelium only a few cells thick (Fig. 14).

Mouse 5 was killed on the 93rd day. A thin layer of lung tissue 5 mm. across was found where the control implant had been put, while in the other leg there was a sarcoma 15 mm. in diameter with a cyst in its midst from the squamous lining of which a growth had arisen that appeared microscopically to be a carcinoma.

Mouse 6, also killed after 93 days, showed a filmy patch of lung tissue as result of the control implantation. There was another patch where *OSSM* had been put with the embryo fragments, but it was nodular because of adenomas scattered through it; and nearby in the same thigh there was a spherical cystic nodule, 10 mm. across, its cavity pink with Scharlach R and full of dead and living papillomatous ingrowth from a thick wall. Histologically the nodule was highly diverse: in some regions what appeared to be a desquamating, squamous cell carcinoma was present, in others a cancer of transitional cell type, and in yet others the rifted, intracanalicular growth already mentioned in the general account of the tumors. Its spaces were lined with proliferating epithelium, cuboidal or more or less flattened, and one to three cells deep.

Pieces of the cyst wall quickly yielded growths on transfer to new hosts, and these were carried through two further groups of mice. In most animals a tumor of transitional type, having a minimal amount of connective tissue, eventually predominated or became the sole entity (Figs. 17 and 18), but some of the growths continued to be complex (Fig. 15), the rifts in them became larger, and the connective tissue between them took on a fibromatous aspect (Fig. 16).

Mice 7, 8, 9, and 10 were killed after 123 days. The embryo fragments implanted in Locke's solution had given rise to shallow patches of lung tissue in excellent condition, yet no larger than in the animals killed earlier. Where *OSSM* had been added to the implants only sarcomas could be found in three instances, but in the fourth a cystic nodule 10 mm. across was present, with a wall consisting of tumor tissue of two widely differing types, ordinary squamous cell carcinomatosis, and a papillomatous neoplasm with narrow connective tissue cores supporting an epithelium one to two cells deep, consisting of cells which differentiated to a pear shape and then came away while still to all appearances in excellent condition (Fig. 19). Bits of the wall gave rise to growths on transplantation and these were propagated in two successive groups of hosts. The same tendency of the supporting connective tissue of the tumor to increase in relative quantity and take on a fibromatous aspect was noted as in mouse 6. During the successive transfers of the tumor the epithelium tended to lose the tall pear shape and become flattened and layered. The tumors of the second generation had nearly the same transitional character as the final tumors of mouse 6. The two remaining mice were killed after 128 days. There were tiny patches of pulmonary tissue at the site of the control implantations, and where *OSSM* had been put a large sarcoma in one instance and in the other a nodule of pulmonary tissue containing two characteristic adenomas.

In sum, sarcomas were more frequent than in the case of epidermal or gastric tissue. The only benign epithelial tumors were pulmonary adenomas, benign papillomas being notably absent. Pieces of two adenomas were transferred to adult hosts, but after 51 and 91 days, respectively (Fig. 6) had done little more than survive. A presumptive carcinoma, found in an implant after 51 days did not succeed on transplantation; it may have been merely metaplastic bronchial epithelium. Three other growths looking like carcinomas, and transferred after longer periods, all did well in new hosts and were carried through several successive groups of them.

The lung tissue from the youngest embryos employed (13 mm. long, 15 days old) yielded growths no different from those derived from the lungs of older embryos.

The First Occurrence of Neoplastic Change

Many of the implants with methylcholanthrene were removed very soon, sectioned serially, and searched for signs of neoplastic change. The alveolar epithelium was found to remain cuboidal during the first 2 weeks, or even longer, but as the alveoli gradually became distended with fluid it flattened and now the adenomas stood forth as discrete spherical entities (Figs. 7 and 8). They remained nearly spherical despite the compression exerted by the thigh muscles, and slowly increased in size. The first one encountered was in an implant 20 days old. Methylcholanthrene had a stimulating effect upon them, as evidenced by the larger size and occasional mitoses in those situated near oil droplets containing the carcinogen.

So completely did the metaplastic activities of the bronchial epithelium simulate malignancy that it proved impossible to tell precisely when carcinomatous changes began. According to the literature mitoses are infrequent in adult lung tissue undergoing metaplasia, but they were numerous when it was taking place in the embryo implants. Where fine droplets of *OSSM* lay scattered amidst the nodules, the metaplastic cells actively invaded and replaced the alveolar tissue and tongues of them frequently extended to outlying droplets situated in the surrounding muscle (Fig. 3). Only when serial section showed that such extension had taken place where there were no droplets could one assume that the cells were independently aggressive and hence presumably malignant. This was first noted after 51 days. Successful transplantation of the growths yielded decisive evidence of their neoplastic character, but when it was unsuccessful one could not be sure that cancer had been absent; for previous tests with the growths arising from epidermis have made plain that some carcinomas which are highly malignant in the original host fail to establish themselves in other hosts of the same homogeneous C strain (1). The longer the time elapsing before transplantation is attempted, the greater seemed to be the likelihood of success, an observation already made as concerns the spontaneous mammary tumors of rabbits (12) and those obtained with methylcholanthrene in embryo epidermis (1). A growth that was morphologically a carcinoma, in an implant 51 days old (Fig. 4) failed on transfer, and so too did another tested after 77 days, whereas success was had with a tumor procured after 81 days and 6 out of 7 succeeded that were tested after 91 to 188 days (Table I). Four of these 6 cancers were carried into further groups of mice in series. On three occasions transfers were made of tissue which the microscope showed to have been merely metaplastic. It gave rise to no nodules.

Effect of Methylcholanthrene on the Implanted Pulmonary Tissue of Adult Mice of the C Strain

In three experiments a suspension of fragments of the lungs of a young adult C mouse was injected, together with *OSSM*, into the thigh muscles of other animals of the same strain and of about the same age. Only the peripheral lung tissue was utilized, to minimize the possibility of carrying bacteria into the graft.

The same technic of injection was employed as with embryo tissue. Serum had been added in one instance to the Locke's solution in which the tissue was suspended, with a view to minimizing injury to the cells, but no better results were obtained than with Locke's solution as such. The 12 implanted mice were killed after 21 to 205 days. Most of the grafted tissue failed to survive, but in animals killed after 21, 28, 44, and 65 days some small patches of it were found. It had not enclosed the *OSSM*, and metaplasia was only once observed. Adenomas were not encountered in serial sections of the 21, 28, and 65 day specimens, but two of them were present in a tiny patch of pulmonary tissue removed 44 days after the implantation. None were present in the lungs of the hosts. Cancers failed to arise in any of the animals though 5 of them were allowed to live for more than 65 days. Seldom was any trace of the implants found. Sarcomatosis had often supervened.

It seems reasonable to ascribe the failure to elicit cancers in these tests to insufficient exposure of the pulmonary cells to the carcinogen. Though some lung tissue survived for more than 2 months, it did not proliferate sufficiently to enclose the *OSSM*, metaplasia did not occur, and the only sign that the carcinogen had influenced it was the development of adenomas. Mice of the C strain usually develop such growths spontaneously as they become old, as is well known (13), and their appearance can be greatly hastened and their number increased in breeds liable to them merely by painting the skin repeatedly with methylcholanthrene (14). Their failure to appear in any considerable number in the lungs of mice receiving *OSSM* together with embryo tissue can be laid in part to the fact that the hosts were still young when killed and in part to the small amount of the carcinogen introduced.

Results with the Pulmonary Tissue of Embryos of the A Strain

Spontaneous adenomas are far more frequent in adult A mice than in those of the C strain (15). Hence the lungs of A embryos were utilized in several experiments. A colony had been raised from individuals supplied by the Roscoe B. Jackson Memorial Laboratory, and random sampling had shown adenomas to be fairly frequent in aging adults.

The general technique was the same as with C embryos, the litters furnishing the lung tissue being about as far along toward birth as those from C females which had been successfully utilized. As usual, some of the implantations were made with Locke's solution only, and some with *OSSM* in the opposite leg of

the host. The control implants did well at first, but later they often became surrounded by great numbers of round cells, and soon after were absorbed. When the tissue had been injected together with *OSSM* it usually enclosed droplets of this latter, metaplasia followed, and adenomas or carcinomas, or both, arose in some instances; but the findings were rendered irregular by local round-cell accumulation and death of the implanted tissue. This phenomenon had been noted previously when the epidermis of A strain embryos was exposed to *OSSM*.

From one of the implants with *OSSM* a cystic nodule had developed by the 125th day and in this there was a malignant papilloma with an invasive base. The growth had a core consisting of large cells resembling ganglion cells (Figs. 20 and 21) which showed mitoses and were obviously neoplastic. They wholly resembled those making up the core of a benign epithelial papilloma induced with methylcholanthrene in an implant of fragments of the squamous portio of the stomach of a C strain embryo (3). Ganglion cells are amongst those elements which do best in ordinary grafts of embryo tissue (16), and it would appear that they had been rendered neoplastic in both the instances now described.

RÉSUMÉ AND DISCUSSION

The lung tissue from C embryos established itself and differentiated to a remarkable extent after transfer to adult hosts of the same strain. It had little capacity for growth, as compared with the fetal cutaneous and gastric tissues, yet sufficient for the implanted fragments to coalesce into a mass within a few days, and after 2 weeks a parenchyma had developed having alveolar spaces of considerable size, lined with flattened epithelium,—as result of distension with fluid, elaborated presumably by the elements lining the bronchi. The spaces were not so large though, nor the cells so flat, as in normal mouse lungs just after birth. No reactive proliferation occurred about the graft but frequently hemorrhage took place into it,—owing perhaps to the trauma of palpation,—and occasionally this was so extensive as to convert it into a little cyst full of blood. More often mere ecchymoses occurred which found expression later in "heart failure cells" plumped out with erythrocytes or brown pigment. Horning has encountered such cells in implants of adult lung tissue with methylcholanthrene crystals (17).

Usually the graft persisted almost unchanged for a long period but occasionally it disappeared after a few months, owing apparently to local accidents, hemorrhage for instance, not to incompatibility of the host. In the case of A mice and Webster-Swiss animals incompatibility was often evident, the grafts dying early amidst a profuse accumulation of round cells; and even in the absence of such accumulation many fared badly. Mice of the Webster-Swiss

breed gave the most irregular results, as might have been expected from their mixed inheritance. The present findings further demonstrated the superiortity of C material. In previous experiments by the same technique the epidermis and gastric tissue of C embryos regularly did well after transplantation, whereas that of A, C3H, and I strain mice often died amidst a profusion of round cells, and successful grafts proliferated poorly as compared with those from C animals (2).

These were the findings after the injection of lung fragments suspended in Locke's solution, with or without Scharlach R in olive oil. In but a single instance did a tumor arise,—an ordinary small adenoma in a graft containing Scharlach R. This dye is known to be weakly carcinogenic, eliciting hepatic adenomas (18), and hence the pulmonary neoplasm cannot be deemed spontaneous. Scharlach R markedly stimulates epidermal cells and under its influence they often grow invasively as if carcinomatous (5), yet it only slightly encouraged proliferation of the lung elements.

Very different were the findings when methylcholanthrene had been added to the olive oil. Considerable growth of the grafted tissue ensued and sizable nodules of it resulted. Reactive connective tissue proliferation took place about these, as also about any outlying droplets of oil. Widespread metaplasia occurred also, mostly of the bronchiolar epithelium but not infrequently of the alveolar lining cells, sometimes of those distant from any bronchioles (Fig. 5). These changes took place irrespective of whether Scharlach R was present. Passey has reported upon a similar alveolar metaplasia in the lungs of rats with chronic bronchiectasis (6), and they provide strong indications, as he remarked, that the cells lining the alveolar wall are epithelial in character, a point long debated. The differentiation taking place secondarily in the cells of many pulmonary adenomas, such that they come to resemble the epithelium of the bronchioles (9), constitutes further evidence to the same effect, for these growths originate from the alveolar lining (8, 9, 19).

The metaplastic changes took the same course as those occurring in the adult lung of several species during pulmonary infections or after the injection of injurious substances (20), and the end result, as in such instances, was a stratified squamous epithelium; but the amount of metaplastic tissue was exceptionally great and the changed cells proliferated actively and were aggressive, often replacing a large proportion of the lung parenchyma (Fig. 4). Sometimes they extended in tongues to oil droplets lying in the muscle round about the graft, and not infrequently they replaced individual muscle fibers. Methylcholanthrene has similar stimulating, attracting and alterative effects on the cutaneous and gastric epithelium of embryos (1, 3), and the findings as a whole suggest that its carcinogenic influence may be exerted on the cell surface and perhaps is physical in nature. So closely did the metaplastic changes

in the pulmonary tissue mimic the carcinomatous that it was often impossible to tell when cancer had put in an appearance and serial sections, even of what appeared to be established squamous cell cancers, were often necessary to determine whether malignancy was really present.

Pulmonary adenomas are not infrequently found in ageing C mice, usually as solitary growths. Animals of the A strain are notably liable to them, and they can be made to appear sooner and in greater number in such animals by painting the skin repeatedly with methylcholanthrene (14). The mere injection of urethane into pregnant C or A mice during the latter half of gestation results in an immediate development of adenomas in the young, these sometimes reaching perceptible size within 3 days after birth (9). In view of these facts there is no ground for surprise at the early development and multiplicity of such growths in grafts of C embryo lung tissue directly exposed to methylcholanthrene. Unlike the carcinomas they were recognizable almost at once after they had begun to form, and hence they appeared to arise earlier than these latter; but whether they really did so is uncertain. None of the cancers seemed to have derived from them, although in adult mice spontaneous malignant adenomas differing but little morphologically from the benign are now and again encountered and the benign ones usually become cancerous after repeated transplantation (21).

Horning has procured tumors of the adult mouse lung by wrapping pieces of the pulmonary tissue around crystals of methylcholanthrene and implanting them in the subcutaneous tissue, with the addition on occasion of stilbestrol (17). Many adenomas and squamous cell carcinomas resulted, as did also two growths which he termed adenocarcinomas, and an anaplastic carcinoma with oat-shaped cells. The growths arose late as compared with those of the present work,—a finding which can be accounted for by the indolent state of the adult pulmonary tissue and by less extensive contact with the carcinogen. There was considerable variation within the primary tumors and an over-all tendency to squamous metaplasia. Horning concluded that all were bronchogenic.

Many of the malignant tumors obtained during the present work were obviously the outcome of a secondary fusion of several carcinomas of very different aspect. Not infrequently they became intermingled and a morphological potpourri resulted. Some of the growths of this sort continued to be complex after transplantation, but when the transfers were kept up one or another of their components eventually outgrew the others and dominated. The carcinomas were remarkably various as a group.

It has been the general assumption that all pulmonary cancers of stratified squamous character, in whatever species, must have arisen from the epithelium of the bronchial tree; but the fact that cells altered by metaplasia not infre-

quently undergo neoplastic change secondarily, when taken in conjunction with the ability of the alveolar lining of the rat and mouse lung to undergo squamous metaplasia, gives cause to doubt this.

Carcinomas of transitional type not infrequently arose from the implants of embryo lung with methylcholanthrene, and on transplantation they retained their character in host after host (Fig. 17). Their occurrence was the more noteworthy because no transitional epithelium exists in the normal lung of the mouse, although it appears temporarily,—but never extensively,—where the cells lining the bronchioles are undergoing metaplasia from the cylindrical to the stratified squamous form. In the cancers just mentioned the course of metaplastic events was abruptly checked and rendered permanent at the transitional stage, as result of the intercurrent neoplastic change.[2]

The prime object of the present experiments, as of the previous ones with embryo skin, stomach, and lung has been to learn how soon in the life of the individual its cells possess the ability to become neoplastic, and how widely distributed and diversified in expression this ability is. Pulmonary tumors have been elicited less consistently than were gastric or cutaneous growths, but this is readily understandable since the olive oil globules containing methylcholanthrene were seldom encapsulated in bulk, but instead were included in the implant only incidentally in most instances, as small globules caught amidst the coalescing lung fragments. Whenever a considerable droplet lay where a bronchiole had been cut across, as now and then happened, the lining epithelium of the latter extended out and around it, just as cutaneous and gastric epithelium is prone to do; and then, as in their case, cancer arose from the wall of the resulting cyst.

The findings stress a fact already made plain by the work with the skin and stomach, that cells procured from mouse embryos in the latter half of development are at least as responsive to the carcinogenic influence of methylcholanthrene as those of adults and undergo neoplastic changes in as wide variety. The results with each of the successive tissues tested have made it more difficult to suppose that these changes can be due to the action of viruses *reaching the cells from without after birth and having effects as narrowly specific as those of the tumor viruses thus far discovered*. As pointed out previously a great multitude of such viruses, all circulating together in the adult host implanted with embryo tissues, would be required to account for the multifarious growths arising from these latter. There would even have to be a virus causing and maintaining transitional cell tumors of the mouse lung, growths never yet encountered under natural conditions. In the case of the lung adenomas ap-

[2] In previous work with the glandular portion of embryo stomachs as affected by methylcholanthrene a transitional cell tumor was also obtained incidentally to induced metaplasia (3), but it was so small as only to be discovered with the microscope.

pearing in the new-born young from mice injected with urethane while pregnant (9), the potentiality to undergo neoplastic change would appear to be possessed by the cells while still *in utero*.

SUMMARY

The lung tissue of mouse embryos of the C strain proliferates to some extent after implantation in adult hosts of the same breed and rapidly differentiates, forming a parenchyma remarkably like the normal. The grafts persist long. When methylcholanthrene dissolved in olive oil has been introduced with them much more growth of them occurs. The carcinogen induces a pronounced metaplasia of the epithelium of the bronchial tree, and the altered cells are often aggressive, multiplying, invading, and largely replacing the parenchyma about them. So closely do they resemble malignant elements in aspect and behavior that it is frequently difficult to tell whether carcinomatous change is not actually present. Genuine tumors soon arise, multiple benign adenomas sometimes appearing within 3 weeks, and indubitable carcinomas a few weeks later. Not a few of the cancers are of transitional cell type, that is to say are expressive of an intermediate stage in the metaplasia.

Under the influence of methylcholanthrene the cells lining the alveolar spaces of the graft sometimes undergo metaplasia also, with result in stratified squamous epithelium. It follows that there is reason to doubt the current assumption that all squamous cell carcinomas of the lung necessarily arise from the bronchial tree. The findings, taken with others previously reported, make it difficult to suppose, furthermore, that the generality of lung tumors can be due to neoplastic viruses entering the organism in postnatal life and having no broader scope than those thus far discovered.

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EXPLANATION OF PLATES

All the sections were stained with eosin and methylene blue. Mr. Joseph B. Haulenbeek made the photographs.

PLATE 6

FIG. 1. Growth found 81 days after implantation of pieces of mouse embryo lung tissue in the thigh muscles of an adult mouse along with Locke's solution. The implanted fragments have proliferated and united to form a tissue with the architecture of adult lung. The several large bronchial structures were lined with tall ciliated columnar epithelium. The rest of the growth is composed of thin-walled aveoli distended with fluid, with some spaces containing large free cells filled with brown pigment ("heart failure cells"). The nodule is wholly unencapsulated, muscle and pulmonary elements lying directly juxtaposed. × 60.

FIG. 2. Section of lung from a mouse embryo near term (20 mm. long). Two bronchioles can be seen lined with columnar epithelium. The alveolar elements are cuboidal. See Fig. 1 for the differentiation occurring after implantation. × 425.

FIG. 3. Growth removed from the muscle 53 days after implantation of embryo lung tissue with methylcholanthrene and Scharlach R in olive oil (OSSM). It illustrates the extreme metaplasia induced by the carcinogen. The many rounded lacunae show where the oil lay. The implanted tissue has undergone extensive metaplasia and most of the nodule consists of stratified epithelium necrosing prior to keratinization. The largest oil depot had been encysted by such epithelium. The arrows point to two spherical growths,—which were benign adenomas as higher magnification showed. They lie amidst compressed alveolar tissue. × 25.

FIG. 4. Mass resulting from the implantation of embryo lung tissue and *OSSM* 51 days previously. Two adenomas can be seen (arrows) lying amidst alveolar tissue. On the left there is a nodule of carcinomatous aspect which may have been merely metaplastic. Transplants failed to survive in new hosts, though one of the adenomas established itself (Fig. 6). × 30.

FIG. 5. Another section through the mass of Fig. 4 to show tufts of squamous epithelium which have originated from the alveolar wall. Lamellated keratin has been produced. The squamous tufts had no direct contact with the bronchioles, as serial sections showed, and often were situated far from them. A typical adenoma lies at the lower right (bracket). × 230.

FIG. 6. Adenoma resulting from the transplantation 91 days previously of a fragment of the growth found in the implant furnishing Figs. 4 and 5. The tumor has done little more than establish itself, but fluid from it has given rise to a small cyst. × 55.

FIG. 7. Adenoma in an implant of embryo lung with *OSSM* examined after 20 days. × 220.
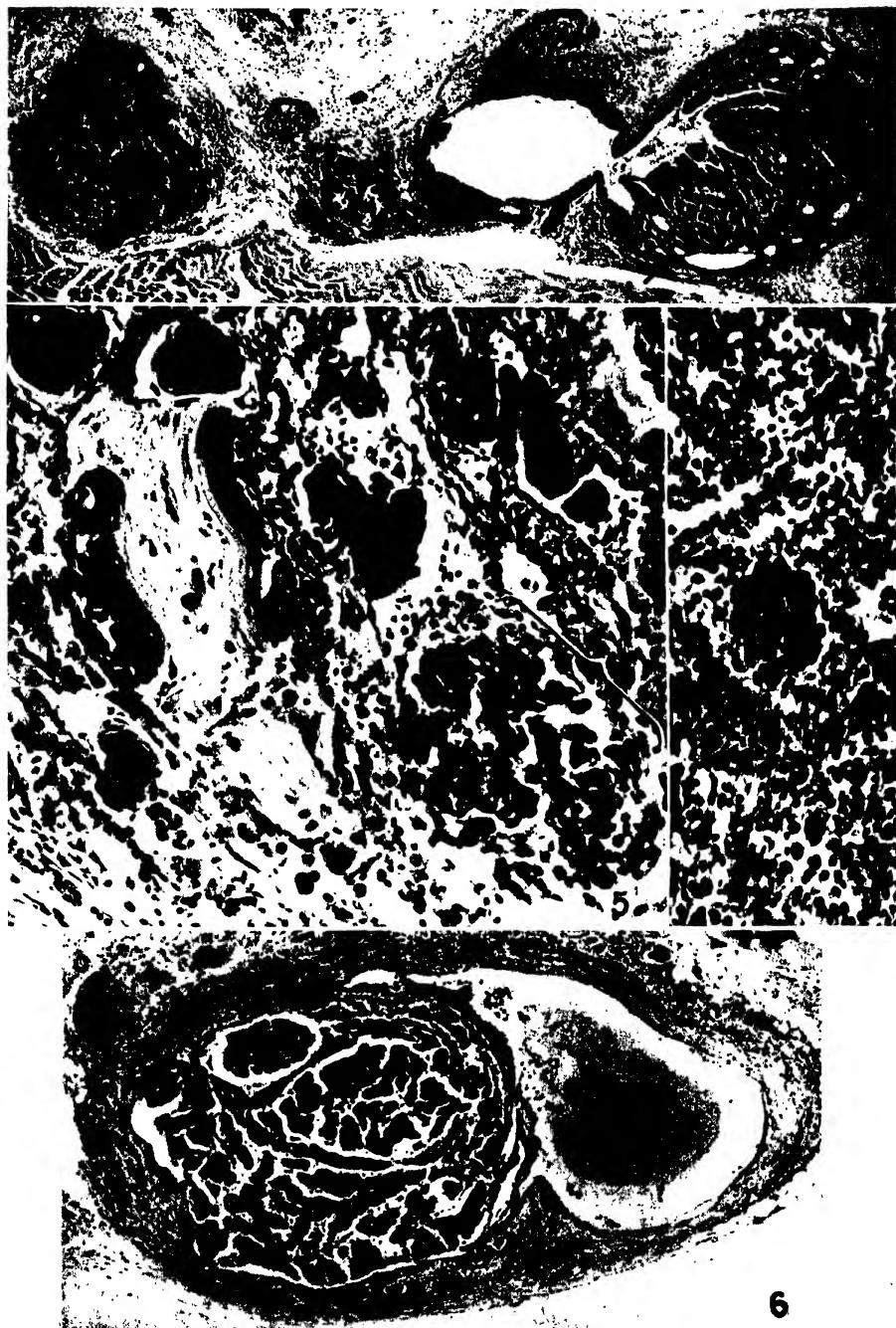
FIG. 8. An adenoma in an implant of embryo lung with *OSSM* examined after 23 days. × 350.

FIG. 9. Nodule of lung tissue removed 57 days after implantation with *OSSM*. Six large adenomas and several small ones are present in the single slice. × 35.

FIG. 10. Section of tumor resulting from the second successive transfer of another adenoma in new hosts. The original growth had been induced in embryo lung tissue implanted in the muscle of an adult mouse along with *OSSM*. The neoplasm is in its sixth serial passage. It has grown progressively in every host, although very slowly, the animals dying after 4 to 6 months with tumors 30 to 50 mm. in diameter. They are for the most part solid and well circumscribed with only small invasive outgrowths into the surrounding tissue, as the present photograph shows. Occasionally they are honeycombed with fluid-filled cysts, and very commonly they contain bone. × 30.

FIG. 11. Higher magnification of the same tumor, in another transplant of the second generation. The cuboidal or columnar cells rarely show mitoses. In this and subsequent generations numerous spicules of dense bone were found. × 125.
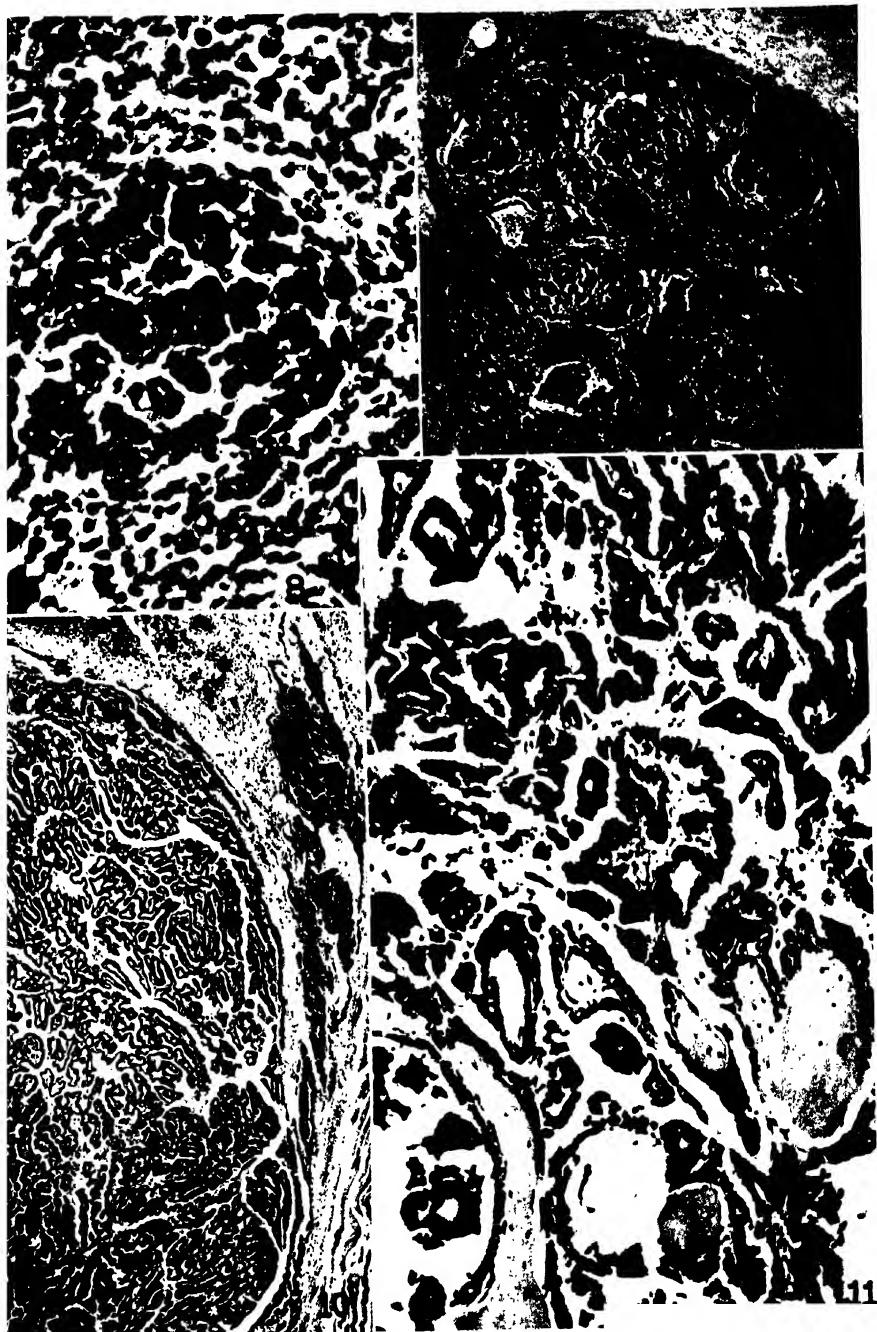
(Smith: Neoplastic potentialities of mouse embryo tissues. V)

Fig. 12. To illustrate various stages of metaplasia. At the center of the photograph the columnar bronchial epithelium is thrown into redundant folds. On the right there is a shallow layer of stratified squamous epithelium, as yet not keratinizing, while on the left there is a thicker layer of transitional epithelium. × 125.

Fig. 13. Part of a nodule 12 mm. across which formed where embryo lung tissue together with *OSSM* had been implanted 81 days previously. An area of alveolar lung tissue lies just below the center of the photograph. The nodule owed most of its size to a cyst full of cellular debris and lined with a carcinoma of papillomatous form. Part of this cyst is shown. The cancer grew rapidly in the 3 successive groups of new hosts to which it was transferred, forming big cysts. × 25.

Fig. 14. Wall of a cyst resulting from third serial transplantation of tissue from the specimen of Fig. 13. The cancer has retained its original morphology. × 35.

Fig. 15. Cross-section through a 10 mm. mass resulting from the initial transplantation of a tumor induced with *OSSM* in embryo lung tissue. Sections of the original growth showed carcinomas of three distinct morphological types. The same three can be seen in the present specimen. The growth occupying the middle of the slice consists of thick septae of fibromatous appearance covered with cuboidal epithelial cells, as higher magnification showed. The clefts between the septae are small. Toward the left there is a carcinoma composed of transitional epithelium heaped up into a layer many cells deep. On the right there is a squamous cell carcinoma. × 17.
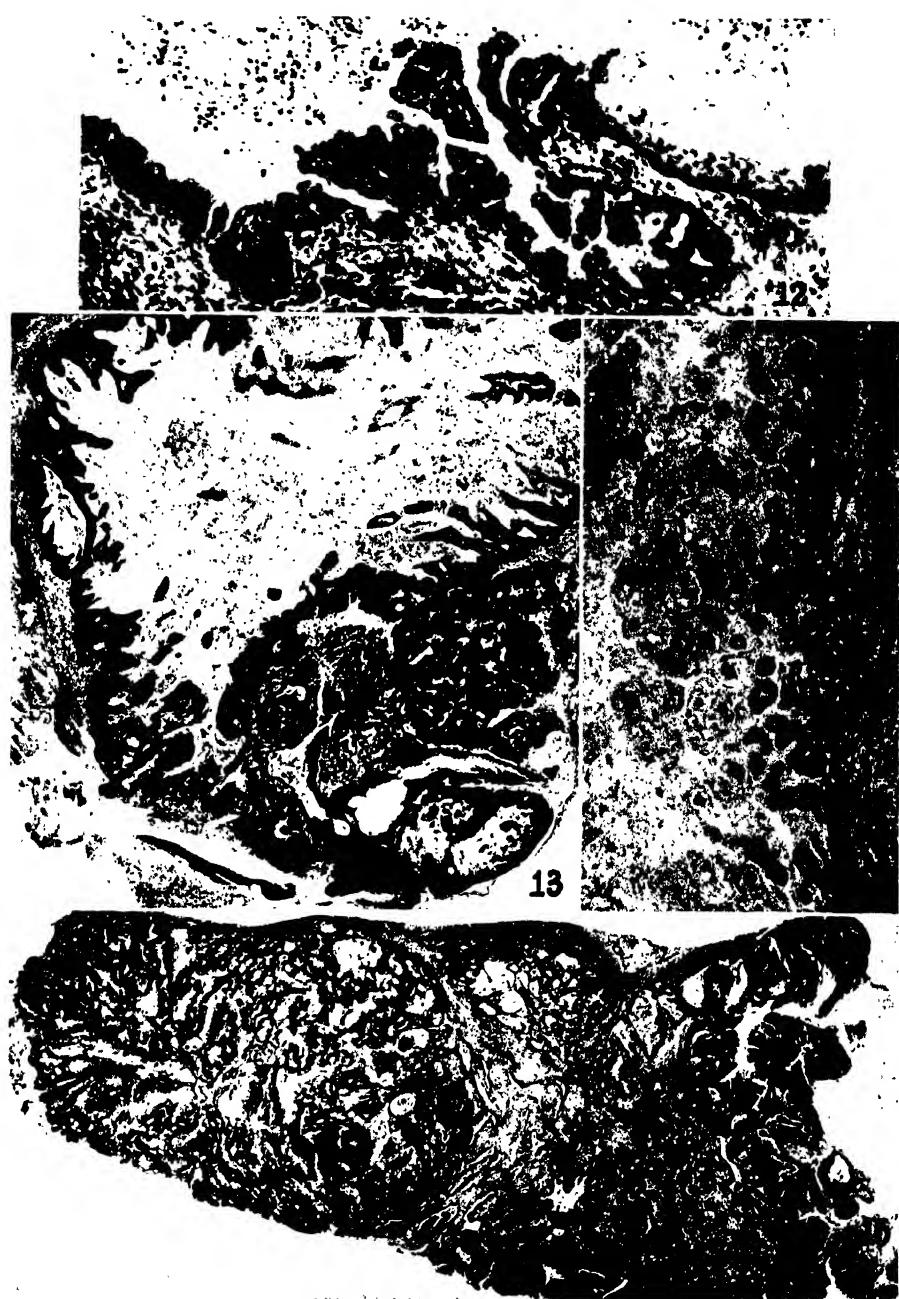
Fig. 16. A tumor resulting from further transplantation (2nd tumor generation) of the growth seen in the middle of the slice of Fig. 15. In the older part of the mass there are numerous clefts separated by thick septae of connective tissue covered with non-keratinizing cuboidal cells in a layer 1 to 2 deep. The vigorously invasive edge of the growth consists of undifferentiated epithelial cells. The tumor was 20 mm. in diameter when its host was killed 38 days after implantation. × 50.

Fig. 17. The transitional cell carcinoma of Fig. 15 growing in an animal of the 3rd tumor generation. Everywhere the neoplasm has the same character. × 30.

Fig. 18. Higher magnification of the transitional cell carcinoma shown in Fig. 17 × 175.
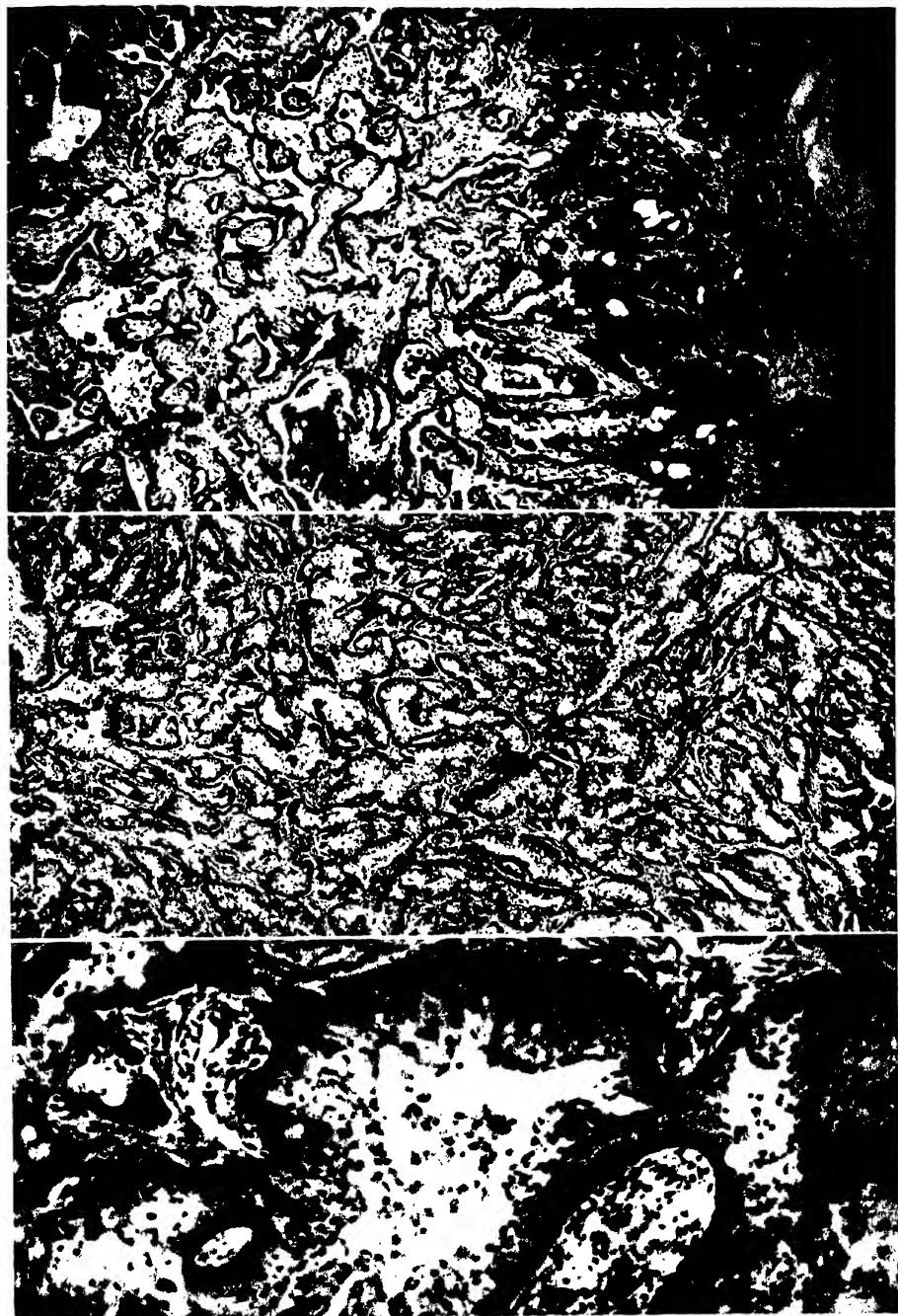
FIG. 19. Alveolar cell carcinoma resulting from the implantation 123 days previously of embryo lung tissue together with *OSSM*. The tumor succeeded on transplantation, but on repeated transfer was eventually encroached upon and destroyed by a transitional cell cancer present with it in the grafts. × 70.

FIG. 20. Section of malignant papilloma, having a core of what appear to be ganglion cells. The growth resulted from the implantation of embryo lung tissue with *OSSM* 125 days previously. × 30.

FIG. 21. Higher magnification of the core, showing the neoplastic character of the cells and a mitosis (arrow). × 350.
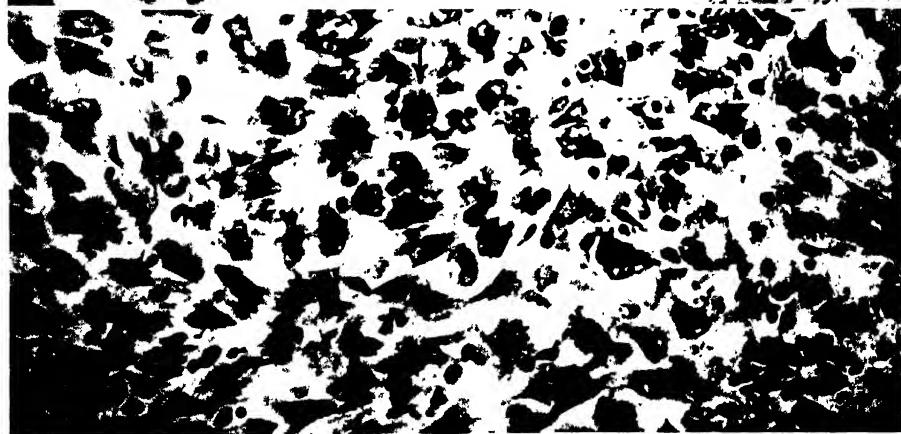
(Smith: Neoplastic potentialities of mouse embryo tissues. V)

PROPERTIES OF A CULTURE OF BCG GROWN IN LIQUID MEDIA CONTAINING TWEEN 80 AND THE FILTRATE OF HEATED SERUM

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INTRODUCTION

For reasons which have been discussed recently in this journal (8), it is probable that many of the irregularities observed in the response of experimental animals and man to the injection of the BCG vaccine stem from the fact that the batches of vaccine used for immunization differ greatly in physiological activity and antigenicity. The present paper describes the first of a series of experimental studies aimed at the development of a vaccine possessing better defined biological characteristics.

As shown elsewhere, one can obtain by the use of the Tween-albumin media young cultures of tubercle bacilli which are sufficiently well dispersed and homogeneous to allow their manipulation by the ordinary bacteriological techniques (4, 5, 6, 7, 9, 11, 12, 14). The dispersed character of the growth resulting from the wetting effect of Tween obviates in particular the necessity of resorting to prior grinding for the preparation of the bacterial suspension. Whereas the clumps of tubercle bacilli harvested from the conventional media contain only a small percentage of viable organisms (18), the "dispersed" cultures obtained in Tween-albumin solutions consist predominantly of living and physiologically active cells. This is demonstrated by the results of quantitative determinations of the numbers of colonies developing on oleic acid-albumin agar (14), and by the high degree of metabolic activity of the bacilli separated from the dispersed cultures (17). Likewise of importance is the fact that a number of standard cultures of mycobacteria which have been maintained for some three years in this laboratory by weekly passage in Tween-albumin media have retained unaltered their morphological and biological properties, and in particular their characteristic level of virulence. Thus, whereas the human strains H37Rv[1] and Jamaica #22[1], and the bovine strains Ravenel[1] and Vallée[1] have remained pathogenic for guinea pigs and mice, the avirulent variants of human strains H37Ra[1], JH16Ra[1], and H4Ra[1] are still completely avirulent for these animal species. Similarly, the attenuated human strain R1Rv[1] and three different strains of BCG have also remained constant in their property of giving rise to discrete pulmonary lesions in mice (following intravenous injection) without being capable of causing progressive disease in normal mice, guinea pigs, or rabbits. Further evidence of the stability of cultures in Tween-albumin media is found in the fact that all cultures

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[1] These strains were originally obtained through the generous cooperation of Dr. Jules Freund (Ravenel and Jamaica strains), Mr. William Steenken (H37Rv, H37Ra, JH16Ra, H4Ra, and R1Rv strains) and Dr. Hubert Bloch (Vallée strain).

mentioned above have maintained their characteristic colonial morphology on oleic acid-albumin agar, the serpentine pattern of growth ("cord formation") which they exhibit remaining unchanged and in direct relation to the level of virulence (7, 15).

It occurred to the writers that these properties of the Tween-albumin media might permit the preparation of cultures of BCG possessing constant and predictable physiological and antigenic characteristics. The findings to be outlined in the present paper describe from this point of view the activity *in vitro* and *in vivo* of a strain of BCG cultivated in a medium in which the filtrate of human serum heated at acid reaction served as a source of albumin.

EXPERIMENTAL

Basal culture medium: The basal medium used was essentially that described in earlier studies for the growth of mycobacteria under submerged conditions (9, 11). As the three BCG cultures so far tested respond particularly well to the dispersing effect of Tween 80, a final concentration of 0.02 per cent of this agent in the medium has been found sufficient to yield stable, dispersed growth. Addition of glucose or glycerin to the basal medium increases the yield of bacterial cells, but may under certain conditions decrease their viability (10). Consequently, these additional sources of energy have been omitted from the medium, as viability was deemed more important than the yield of bacterial cells in experiments aiming at the establishment of immunity.

The medium was prepared as follows:

KH_2PO_4	1.0 Gm.
$Na_2HPO_4 \cdot 12H_2O$	6.3 Gm.
Asparagin	2.0 Gm.
Tween 80	0.2 Gm.
Enzymatic digest of casein	0.5 Gm.
Distilled water	900 cc.

Add:

Ferric ammonium citrate	0.05	Gm.
$MgSO_4 \cdot 7H_2O$	0.01	Gm. (1 cc. of a 1 per cent stock solution in distilled water)
$CaCl_2$	0.0005	Gm. (1 cc. of a 0.05 per cent stock solution in distilled water)
$ZnSO_4$	0.0001	Gm. (1 cc. of a 0.01 per cent stock solution in distilled water)
$CuSO_4$	0.0001	Gm. (1 cc. of a 0.01 per cent stock solution in distilled water)

Adjust pH to 6.5–6.8

The medium was distributed in 25 cc. amounts in Erlenmeyer flasks of 250 cc. capacity and was autoclaved at 15 lbs. pressure for 15 minutes.

The use of filtrate of serum heated at acid reaction as source of albumin: It has been previously shown that the albumin fraction of serum (Plasma Fraction V of the alcohol fractionation method developed by Cohn, *et al.*) exerts on tubercle bacilli a growth-enhancing effect which depends upon two independent mechanisms: (a) Fraction V protects the bacilli against a variety of toxic influences; and (b) supplies them with some accessory growth factor, as yet unidentified (4,

6, 7, 9, 11). In the past, the Fraction V of bovine plasma[2] has been used almost exclusively as a source of albumin. Unfortunately, this material is costly and not readily available to workers outside the United States. Moreover, as BCG cultures may be used for vaccination of human beings, it appears undesirable to add protein of animal origin which might act as antigen to the culture media in which the bacteria are grown. Consequently, an attempt has been made to find an inexpensive and convenient method for the separation from human serum of a fraction which would have the same growth-promoting properties as the Fraction V separated from plasma by the Cohn method.

The technique which has given the best results in this laboratory is based on the fact that, at acidic reactions, serum albumin can be heated to high temperatures without undergoing denaturation and without losing the associated properties responsible for the enhancement of growth of tubercle bacilli. The serum globulins, on the contrary, become completely denatured under the same conditions. The technique is carried out as follows:

Serum is acidified to pH 2.0 to 2.5 by the addition of normal HCl (approximately 0.5 to 0.8 cc. of normal acid per 10 cc. of serum). It is then heated at 65° to 70°C. for 20 minutes. The heated serum, which should remain fluid and transparent, is cooled and brought back to pH 6.5 by the careful addition of 0.1N sodium hydroxide. An abundant precipitate separates, which consists chiefly of denatured globulins, while a large part of the albumin remains in solution. The denatured insoluble proteins are then removed by filtration through filter paper or by centrifugation. In some instances, however (and particularly in the case of human sera), the precipitate is of such a fine colloidal nature as to prevent separation. Under these circumstances, separation may be effected by the addition of a small amount (approximately one-tenth the volume) of a water-immiscible organic solvent (chloroform or ether, for example) to the heated serum which has been brought back to pH 5.0. Gentle agitation causes immediate agglutination of the precipitate which then can be readily separated from the soluble part by filtration.

Sterilization of the filtrate can be carried out by a second filtration through bacteriological porcelain candles. In this laboratory the candles are first cleaned with water, then baked in an electric oven at 560°C., and washed again with water prior to sterilization. Although it is possible and convenient to sterilize albumin solutions by filtration through asbestos pads of the Seitz type, care must be taken that the pads are thoroughly washed before use as they often release impurities which interfere with the growth of tubercle bacilli. It is advisable to heat the sterilized filtrate up to 55°C. for 20 minutes in order to distill off the traces of organic solvent left after removal of the precipitate of denatured proteins.

The concentration of albumin in the filtrate of heated serum can be determined by precipitation with 5 per cent trichloracetic acid or 2.5 per cent sulfosalicylic acid. In our experience, approximately 60 per cent of the original albumin in the serum is recovered in the filtrate by the technique outlined above, whether the filtrate be prepared from the serum of man, ox, horse, or rabbit.

In the present experiments, the filtrate of heated human serum was added aseptically to the autoclaved basal medium in amounts of 5 cc. (of filtrate at pH 6.5) for 25 cc. of medium.[3]

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[2] Produced commercially by the Armour Laboratories, Chicago, Illinois.

[3] Many of the experiments described in the present report were carried out with samples of filtrates of human heated serum received through the generous cooperation of Mr. C. E. Bender of Microbiological Associates, Flemington, New Jersey.

The BCG Culture

Although the three different strains of BCG available in our laboratory have been cultivated successfully in the medium described above, all experiments to be reported here have been carried out with one single strain (Phipps). It was received through the courtesy of Dr. Joseph Aronson and is used for the preparation of BCG vaccine at the Henry Phipps Institute.

Flasks containing approximately 30 cc. of the medium prepared with Tween and serum filtrate were inoculated by adding 3 cc. of the BCG culture grown for eight days at 37° C. in the same medium. After one week of incubation, the new growth was very abundant and well dispersed. It was seen on the bottom of the flask as a fine sediment which could readily be resuspended by gentle manual shaking to give a macroscopically homogeneous suspension. Microscopic examination revealed the presence of clumps of various dimensions (some containing probably up to one thousand bacilli) in addition to many smaller clumps and isolated bacterial cells. The bacilli took the acid-fast stain but the cells were somewhat shorter than those present in a sample of vaccine prepared from the same strains by the conventional technique at the Henry Phipps Institute (1a).

Colonial morphology: In order to study the colonial morphology of the cultures growing in Tween-serum filtrate medium, dilutions of these cultures in 0.1 per cent albumin in saline were spread on the surface of oleic acid-albumin agar in Petri dishes, the albumin used for the dilutions and the preparation of the agar medium being Armour's bovine plasma Fraction V (11, 14). Colonies of uniform morphology developed, which exhibited some tendency to spread over the surface of the agar medium. The serpentine pattern of growth was less pronounced than in the case of the fully virulent cultures but was nevertheless obvious enough to allow a ready distinction between the BCG colonies and the raised opaque colonies of the fully avirulent variants (15). The comparative colonial morphology of the three culture types is illustrated in figure 1.

Virulence for animals: The culture of BCG grown in liquid medium containing Tween and the filtrate of heated serum retained its characteristic degree of attenuation for experimental animals. Albino mice, five weeks old, weighing approximately 20 Gm., were injected into the tail vein with 0.2 cc. of undiluted culture. The animals remained in apparent good health and continued to gain weight, although many discrete, nonprogressive pulmonary lesions were seen when they were sacrificed two months later. Guinea pigs inoculated intramuscularly with 0.1 cc. of the same undiluted culture also continued to gain weight and failed to reveal macroscopic tuberculous lesions when sacrificed after three months. These results become the more significant in view of the fact that the cultures used for the virulence tests contained many more living bacilli (from one hundred to ten thousand times more per unit volume) than the bacterial suspensions used as vaccines in the conventional procedures. No information is available concerning the response of human beings to cultures of BCG grown in media containing Tween and the filtrate of heated human serum.

Viability of the Cultures

It had been found in earlier studies from this laboratory that well-dispersed cultures of tubercle bacilli in Tween-bovine albumin medium were capable of initiating growth regularly with inocula of 10^{-8} cc. and occasionally with 10^{-9} cc.,
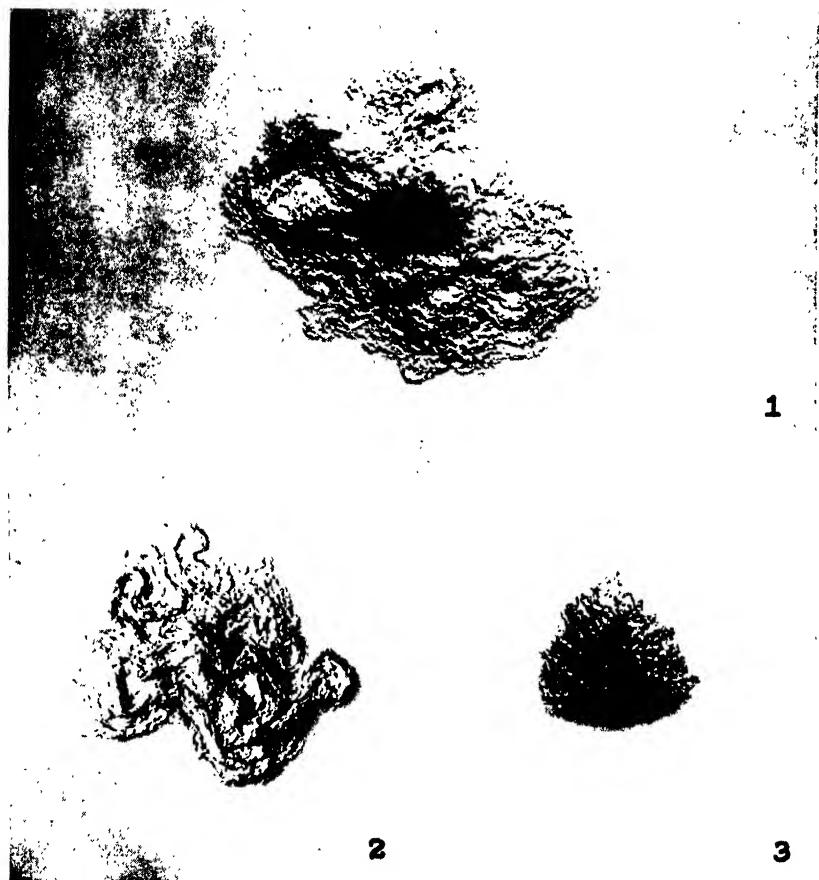

FIG. 1. Comparative colonial morphology of three strains of tubercle bacilli:
(1) BCG (strain obtained from the Henry Phipps Institute)
(2) H37Rv (virulent human strain)
(3) H37Ra (avirulent variant of H37Rv)
All colonies are from a 9-day growth on oleic acid-bovine albumin agar. × 230.

a finding which suggested that most of the cells in the cultures were viable (4, 9). This conclusion has received much support from more recent investigations based on the statistical analysis of the number of colonies developing on the surface of oleic acid-albumin agar plates inoculated with proper dilutions of cultures in liquid Tween-bovine albumin medium (14). Similar studies carried out with cultures of BCG, cultivated in media containing the filtrate of heated human serum

in lieu of bovine albumin, have yielded similar results and have led to the following conclusions:

Cultures of BCG grown as described earlier in this report reach in eight days a density and a bacterial population corresponding to approximately three-fourths of the maximal population of viable bacteria to be obtained on further incubation. The maximum level is reached after two to three weeks of incubation.

Addition to the medium of 0.1 per cent glucose approximately doubles the final density of the culture and the bacterial population (as measured by the number of colonies growing on oleic acid-albumin agar). Still higher bacterial counts are obtained during the first week of incubation if the amount of sugar is increased up to 0.5 per cent. With this higher sugar content, however, there soon occurs on further incubation a sharp fall in the number of viable cells although the turbidity of the culture does not decrease at the same time. Microscopic study reveals that many of the bacilli are then undergoing morpholog-

TABLE 1

Viability of BCG (Phipps) Culture in Liquid Medium Containing Tween and the Filtrate of Heated Human Serum

PREPARATION	INCUBATION 37°C.	STORAGE (ICEBOX)	COLONIAL COUNT* (PER CC. OF CULTURE)
I	8 days	0	20×10^6
II	8 days	0	50×10^6
II	8 days	3 weeks	19×10^6
III	8 days	0	61×10^6
III	8 days	6 weeks	54×10^6

* Average of counts made on 5 plates of oleic acid-albumin agar. Colonial counts do not describe exactly the number of viable cells as the bacterial suspensions contain many microscopic clumps in addition to single cells.

ical alteration and are losing the acid-fast property. Similar or even more pronounced loss of viability has been observed when glycerin was substituted for glucose in these experiments (10).

Cultures (without glucose) incubated for eight days at 37° C., and then placed in the refrigerator at 4° to 7° C. for periods of three or six weeks, do not exhibit any detectable alteration in cellular morphology or any decrease in viability during storage. Longer periods of refrigeration were not tested.

Some of the findings concerning the effect of storage on viability are recorded in table 1. It must be kept in mind that the number of living cells present in the cultures is considerably larger than would appear from the number of colonies, as the cultures in Tween-serum filtrate medium contain many microscopic clumps in addition to isolated organisms.

Response of Guinea Pigs to the Injection of BCG Cultures Grown in Media Containing Tween and the Filtrate of Heated Human Serum

Although cultures of BCG grown in Tween-serum filtrate medium appear unable to cause progressive disease, it is likely that they can multiply to some ex-

tent in the body of normal guinea pigs. This fact is strongly suggested by the results presented in table 2, where it is shown that injection of very small amounts of culture (0.000001 cc.), containing but a few infective units, was sufficient to sensitize guinea pigs to tuberculin.

It will be noted that sensitization was also established following the injection of 0.1 cc. of cultures kept for three or six weeks in the refrigerator. The desirable quantitative data are not yet available to establish whether, during refrigeration, sensitizing ability had been retained to the same degree as viability.

TABLE 2

Sensitization of Guinea Pigs with Fresh and Old Cultures of BCG in Liquid Medium Containing Tween and the Filtrate of Heated Human Serum

BCG			PPD†	TUBERCULIN REACTION
Preparation*	Storage (icebox)	Amount injected		
		cc.	*mg.*	
I	0	0.1	0.0005	++++
I	0	0.1×10^{-5}	0.0005	++
I	0	0.1×10^{-6}	0.0005	0
II	3 weeks	0.1	0.0005	++++
III	6 weeks	0.1	0.0005	++++
Medium	0	0.1	0.005	0

* Incubated at 37°C. for 8 days (see table 1).

† PPD (0.1 cc. of solution in saline) injected intracutaneously 6 weeks after vaccination with BCG culture. Readings made 24 and 48 hours later.

An experiment was instituted to test the effect of vaccination with BCG on the resistance of guinea pigs to the subcutaneous injection of virulent human bacilli. The animals, divided into 7 groups, were treated as indicated on page 73.

A detailed account of the fate of the individual animals in the seven different groups is now in course of preparation and will be published elsewhere (13). Only a brief summary of the general trend of the results will be presented here.

All animals which had received the BCG culture alone (groups IB and IIB) continued to gain weight at a normal rate, and did not reveal any macroscopic tuberculous lesions on examination post mortem.

All animals infected with the virulent human strain without prior vaccination (group V) began to lose weight within two weeks after infection and reached an extreme degree of emaciation. Ten had already succumbed when the experiment was terminated nine weeks after infection, and all survivors were found to have generalized tuberculosis of such an extent that they probably would have died soon thereafter.

The animals of the four other groups (which received both the BCG culture and the challenge infection) exhibited a fairly uniform pattern of response. Most of them continued to gain in weight after the challenge infection, although some-

what more slowly than the control animals. Only a few (approximately 10 per cent) exhibited signs of emaciation and only one died of tuberculosis. Post-mortem examination revealed macroscopic tuberculous lesions in all animals. In general the lesions were limited to a few tubercles in the spleen and liver, but in some cases they were widely distributed throughout the tissues. These were the same animals which showed severe emaciation. Thus, all four preparations

EXPERIMENTAL GROUPS	NUMBER OF GUINEA PIGS	BCG VACCINATION	CHALLENGE INFEC-TION (SUBCUTANEOUS ROUTE) 7 WEEKS AFTER VACCINATION
IA	16	0.1 cc. of vaccine prepared at the Henry Phipps Institute.†	0.1 cc.‡
IB	8	0.1 cc. of vaccine prepared at the Henry Phipps Institute.† .	0
IIA	17	0.1 cc. of fresh culture in Tween-human serum filtrate medium.*	0.1 cc.‡
IIB	8	0.1 cc. of fresh culture in Tween-human serum filtrate medium.*	0
III	17	0.1 cc. of culture in Tween-human serum filtrate medium* stored for 3 weeks in ice-box.	0.1 cc.‡
IV	17	0.1 cc. of culture in Tween-human serum filtrate medium* stored for 6 weeks in ice-box.	0.1 cc.‡
V	16	0	0.1 cc.‡

* All these cultures were incubated at 37°C. for 8 days.

† This BCG vaccine, prepared according to the classical technique (1a), was shipped from Dr. Aronson's laboratory immediately upon completion, and injected the next day into the experimental guinea pigs at The Rockefeller Institute.

‡ Undiluted culture of human strain of tubercle bacillus (Amerzanga) incubated for 8 days in Tween-bovine albumin medium. This culture was kindly sent us by Dr. Aronson.

of vaccine elicited a demonstrable degree of protection against infection by the subcutaneous route, but the results did not reveal any convincing difference between them. It is possible, however, and indeed is considered likely, that differences could have been brought out by changing the design of the experiment in regard to such factors as: the amount of vaccine used, the size of the challenge dose, the time elapsed between vaccination and challenge, and the route of infection.

DISCUSSION

The results of the experiments which have just been outlined seem to show that the BCG culture, which had grown diffusely and rapidly below the surface of the medium containing Tween and the filtrate of heated human serum, had retained its characteristic biological properties. It failed to establish progressive disease in mice and guinea pigs; it was capable of converting guinea pigs to the tuberculin-positive state; and it increased their resistance to artificial infection (by the subcutaneous route) with virulent human tubercle bacilli.

Needless to say, the present data do not establish that BCG vaccination brought about a true state of immunity, for the introduction of the challenge-infecting dose by the subcutaneous route did not allow adequate differentiation between the effect of allergy and immunity (8). The results demonstrate only that the culture grown in the Tween-serum filtrate media compared well qualitatively with the vaccines prepared by the conventional technique (1a, 1b) and may even present definite advantages over the latter from the quantitative point of view. The content of viable, physiologically active cells in the culture is extremely high, as shown by colony counts and by the ability of very minute amounts of culture to elicit the tuberculin-positive state in guinea pigs. Moreover, the number of viable cells is predictable and remarkably stable even after prolonged periods of incubation or of storage. This stability should greatly facilitate the planning of immunity experiments and the analysis of their results.

Other minor advantages of the dispersed cultures deserve brief mention, namely: the rapidity with which they can be obtained; the fact that they require for their preparation only standard bacteriological techniques; the elimination of the transfer operations and of the necessity of grinding the bacterial growth before distribution of the final product; and their stability in the very medium in which they are grown which allows ample time for the performance of the control tests required of all biological products. Nevertheless, it is obvious that the technique of cultivation of BCG outlined in the present report should be regarded only as an experimental procedure and not as one recommended for the preparation of the vaccine. It constitutes, however, a demonstration that one can obtain by adequate cultural methods bacterial suspensions of dependable physiological and antigenic activity.

SUMMARY

A simple technique is described for the preparation from human and animal sera heated at acid reactions of a filtrate which can replace the albumin fraction for the cultivation of tubercle bacilli.

Cultures of BCG grow rapidly and diffusely in a liquid medium containing this crude albumin fraction of human serum and a small amount of the wetting agent Tween 80.

The cultures obtained under these conditions consist predominantly of cells which are viable and physiologically active. Quantitative colonial counts reveal that the bacilli retain their viability for prolonged periods of time under ordinary conditions of refrigeration and even at 37° C.

Cultures of BCG grown diffusely in media containing Tween 80 and the filtrate of heated human serum retain unaltered their characteristic degree of attenuated virulence and their ability to elicit in guinea pigs both tuberculin allergy and an increased resistance to subcutaneous infection with virulent human tubercle bacilli.

It is possible to sensitize guinea pigs with very minute amounts of dispersed culture (0.000001 cc.) containing only a few viable units. The homogeneity and stability of the cultures, even after prolonged periods of storage, greatly facilitate

the operations required for the preparation, distribution   biological control
of the vaccine.

SUMARIO

*Propiedades de un Cultivo de BCG Sembrado en Medios Líquidos que Contenían
Tween 80 y el Filtrado de Suero Calentado*

La sencilla técnica descrita es para la preparación, de suei os h nanos y ani-
males calentados a reacciones ácidas, de un filtrado que suplante la fracción de
albúmina en el cultivo de bacilos tuberculosos.

Los cultivos de BCG crecen rápida y difusamente en un medio líquido que
contenga la fracción de albúmina bruta del suero humano y una pequeña canti-
dad del humectante Tween 80.

Los cultivos obtenidos en esas condiciones constan predominantemente de
células viables y fisiológicamente activas. Las numeraciones cuantitativas de
las colonias revelan que los bacilos retienen su viabilidad por períodos pro-
longados de tiempo en las condiciones ordinarias de refrigeración y hasta a
37°C.

Los cultivos de BCG sembrados difusamente en medios que contienen Tween
*80 y el filtrado de suero humano calentado retienen, sin alteración, su caracte-
rística virulencia atenuada y su capacidad para evocar en los cobayos tanto
alergia a la tuberculina como mayor resistencia a la infección intramuscular
por bacilos tuberculosos humanos virulentos.*

Resulta posible sensibilizar a los cobayos con cantidades pequeñísimas
(0.000001 cc.) de cultivo disperso, que sólo contengan algunas unidades viables.
La homogeneidad y estabilidad de los cultivos, aun después de prolongados
períodos de conservación, facilitan considerablemente las operaciones requeridas
para la preparación, distribución y control biológico de la vacuna.

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PRODUCTION OF BCG VACCINE IN A LIQUID MEDIUM CONTAINING TWEEN 80 AND A SOLUBLE FRACTION OF HEATED HUMAN SERUM

I. PRODUCTION AND VIABILITY OF THE CULTURE

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Adequate biological or immunological criteria are still lacking for the standardization of the vaccines made from living attenuated bovine tubercle bacilli (BCG). It is probable that, eventually, standardization will be based on quantitative measurements of the humoral or cellular response of the host to the specific bacterial antigen which induces protective immunity against tuberculous infection. As long as the mechanism of antituberculous immunity remains unknown, however, it may be necessary to standardize the vaccine in terms of less specific criteria: the level of attenuation of the BCG culture, the amount of bacterial material present in the vaccine preparation, the viability and physiological activity of the cells which it contains, etc. All these different characteristics are probably of importance in determining the extent to which BCG is capable of multiplying *in vivo*, and its effectiveness in establishing tuberculin allergy and resistance against infection. In the hope of minimizing both qualitative and quantitative variations in the activity of the vaccine, most BCG workers have advocated a rigid adherence to the preparative techniques worked out by Calmette and his associates (1, 2). It is almost certain, however, that, despite all precautions taken in the preparation of the culture and in the distribution of the vaccine, the batches of final product differ greatly in biological activity.

According to the classical technique, the BCG culture must be grown on the surface of some synthetic medium (Sauton for example) where it forms a pellicle of varying but not inconsiderable thickness (1, 3). This mode of growth results in marked heterogeneity of physiological environment since the ease of gaseous exchanges and of access to nutrients are naturally influenced by the position of the bacterial cells with reference to the atmosphere and to the fluid medium. Removal of the bacterial pellicle from the nutrient solution and the preparation by grinding of a fine bacterial suspension are procedures that

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introduce further elements of heterogeneity and that bring about the death of many of the microorganisms. Loss of viability probably occurs as a result of the physical trauma caused by grinding and of the physiological disturbances following upon the sudden changes of environment to which the bacterial cells are exposed. It is well known, furthermore, that the BCG cells which have survived the operations inherent in the preparation of the vaccine soon begin to die in the fluid used for distribution of the final product. This fact has been acknowledged in the recommendation that the vaccine be used within a very few days following its preparation. Some figures may be in order to give concrete meaning to these general observations. The standard BCG vaccine, as distributed, contains approximately 10^9 bacterial cells per cc. (as determined by direct microscopic count); quantitative determinations carried out in our laboratory indicate that, within 48 hours after it has been issued, the vaccine contains at the most only 10^4 to 10^5 viable units per cc. and that its viability continues to decrease rapidly during the following few days. Thus, the vaccine contains at best only a small fraction of 1 per cent of viable cells at the time of its use.[1] Under these conditions, it is entirely meaningless and indeed misleading to prescribe the dose of vaccine in milligrams or cubic centimeters, as these weight and volume units give no idea whatever of the number of living organisms in the product.

In order to facilitate standardization of the final product, attempts are being made in several places to stabilize the vaccine by desiccation *in vacuo*. It is well to realize that no method of desiccation—however effective and dependable—can correct the elements of heterogeneity resulting from the procedures presently in use for the preparation of the culture and of the bacterial suspension. The purpose of this and the following paper is to show that one can obtain BCG cultures consisting of a very large percentage of living bacterial cells; and that these cultures retain for several weeks at least their viability and the power to induce in guinea pigs both tuberculin allergy and increased resistance to infection with virulent bacilli.

<div align="center">EXPERIMENTAL</div>

Cultures.—The experiments on cultivation of BCG were carried out with four cultures available in the United States, and with one obtained from Copenhagen (through the courtesy of Dr. J. Holm) in the form of a sample of standard vaccine. As the five cultures exhibited essentially the same behavior in preliminary experiments, only one of them was studied more extensively. This culture (labelled ((Phipps)) in our collection) was received on May 21, 1947, from Dr. Joseph Aronson of the Henry Phipps Institute in Philadelphia.[2] It has since been

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[1] It must be realized that the number of viable units does not represent the number of viable cells as the vaccine contains many microscopic clumps, each one of which gives rise to only one colony while consisting of many living organisms.

[2] We have been informed by Dr. Aronson that he had received this culture from the Pasteur Institute of Paris under the code number BCG Number 793, series 2 (February 21, 1946).

maintained in our laboratory in the Tween-bovine albumin medium described previously (4) by transferring, at 1 to 3 week intervals, one drop of culture to 5 cc. of the same liquid medium and incubating at 37°C. for 8 days. At the end of the incubation period, the culture is placed in the refrigerator (at 4–5°C.) until needed.

As far as can be judged, the BCG culture Phipps has retained unaltered the morphological and biological characteristics that it possessed when first received from Dr. Aronson. Thus, it exhibits on oleic acid–bovine albumin agar a distinct colonial morphology intermediate between the spreading "serpentine" type of the fully virulent strains and the unoriented, heaped appearance of the completely avirulent variants (5, 6). Its virulence also appears to have remained constant; 0.2 cc. of a 7 to 10 day old culture in Tween-bovine albumin medium, injected into the tail vein of mice, gives rise to discrete pulmonary lesions but fails to cause progressive disease and death (7).

In the final stages of the present study, the Phipps culture described above was compared in a few experiments with a new subculture of the same strain received in the form of a sample of standard BCG vaccine again from Dr. Aronson (in March, 1949). As far as could be judged from the limited number of observations made (type of growth in liquid and on agar media, response of guinea pigs to the intracutaneous injection of serial dilutions of cultures grown in liquid Tween-serum filtrate medium (8)), the two subcultures could not be differentiated either *in vitro* or *in vivo*.

Methods Used for Measuring the Viability of Cultures.—The numbers of viable cells (or clumps of cells) were determined by enumeration of colonies on oleic acid–bovine albumin agar, using techniques which have been described in detail elsewhere (4, 9). All culture dilutions were made in 0.1 per cent bovine albumin in distilled water. In a few cases the dilutions used for plating were inoculated also into 5 cc. of liquid Tween-bovine albumin medium (4) distributed in 5 cc. amounts in tubes 25 mm. in diameter. Readings of the extent of growth (colonial counts or evaluation of turbidity) were made at weekly intervals.

Production of Dispersed Growth of BCG in Liquid Medium.—As pointed out in earlier publications, it is possible to obtain submerged and diffuse growth of tubercle bacilli by adding to ordinary synthetic media certain wetting agents and the albumin fraction of serum. An attempt was therefore made to adapt this cultural technique to the production of BCG vaccine.

The basal medium was the same as that previously described (4). It did not contain either glucose or glycerine. Tween 80 was added in a final concentration of 0.02 per cent before autoclaving.

In the past, we have used almost exclusively as source of albumin the fraction V separated from bovine plasma by alcohol precipitation.[a] Unfortunately, this material is not readily available to workers outside the United States. Moreover, as BCG cultures may be used for vaccination of human beings, it appears undesirable to add to the culture media in which they are grown any protein of animal origin that might act as antigen. We have attempted therefore to work out an inexpensive and convenient method for the separation from human serum of a fraction that would have the same growth-promoting properties as the fraction V separated from plasma by the Cohn method.

The technique which has given the best results in our laboratory is based on the fact that, at acidic reactions, serum albumin can be heated to high temperatures without undergoing denaturation and without losing the associated properties responsible for the enhancement of

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[a] Bovine plasma fraction V is available commercially from Armour Laboratories, Chicago.

growth of tubercle bacilli. The serum globulins, on the contrary, become completely denatured under the same conditions. The technique is as follows:—

Serum, diluted with an equal volume of physiological saline, is acidified to pH 2.0–2.5 by the addition of normal HCl (approximately 0.5 to 0.8 cc. of normal acid per 10 cc. of serum). It is then heated at 65–70°C. for 20 minutes. The heated serum, which should remain fluid and transparent, is cooled, and brought back to pH 6.5 by the careful addition of 0.1 N sodium hydroxide. There separates an abundant precipitate consisting chiefly of denatured globulins, while a large part of the albumin remains in solution. The denatured insoluble proteins are then removed by filtration through filter paper or by centrifugation. In some cases, however (and particularly is this so in the case of human sera), the precipitate is of such a fine colloidal nature as to prevent separation. Separation may then be effected by adding to the heated serum brought back to pH 4.5–5.0 a small amount (approximately one-tenth the volume) of a water-immiscible organic solvent (chloroform or ether for example). Gentle agitation causes immediate agglutination of the precipitate which can then be readily separated from the soluble part by filtration.

Sterilization of the filtrate can be carried out by a second filtration through bacteriological porcelain candles. In our laboratory these are first cleaned with water, then baked in an electric oven at 560°C., and washed again with water before being autoclaved. Although it is possible, and convenient, to sterilize albumin solutions by filtration through asbestos pads of the Seitz type, care must be taken that the pads are thoroughly washed before use as they often release impurities which interfere with the growth of tubercle bacilli. It is advisable to heat the sterilized filtrate at 55°C. for 20 minutes in order to distill off the traces of organic solvent left after removal of the precipitate of denatured proteins.[4]

The concentration of albumin in the filtrate of heated serum can be determined by precipitation with 5 per cent trichloracetic acid or 2.5 per cent sulfosalicylic acid.

The basal medium was distributed in 25 cc. amounts in Erlenmeyer flasks of 125 cc. capacity and autoclaved. To each of these flasks was also added 5 cc. of the filtrate of heated human serum prepared as described above.[5]

Each flask was inoculated with 3 cc. of BCG culture grown for 7 to 10 days in the same medium.

After incubation for 1 week at 37°C. the new growth could be seen on the bottom of the flask as an abundant fine sediment that could be readily resuspended by gentle manual shaking to give a macroscopically homogeneous suspension. Microscopic examination revealed the presence of clumps of various dimensions (some containing probably up to one thousand bacilli) in addition to many smaller clumps and isolated bacterial cells. The bacilli were uniformly acid-fast but the cells were somewhat shorter than those present in a sample of vaccine prepared from the same strain by the conventional technique at the Henry Phipps Institute.

[4] There is no evidence that heating at acid reaction and filtration after neutralization will exclude the filterable viruses—the serum hepatitis virus for example—that may be present in the original sample of human serum. Should the method described in the present paper come to be used in the preparation of vaccine for injection into human beings, it would be advisable to obtain the heated serum filtrate from a serum sample proved to be free of serum hepatitis virus, or rendered free of it by irradiation or by treatment with nitrogen mustard.

[5] Many of the experiments described in the present report were carried out with samples of filtrates of heated human serum received through the generous cooperation of Mr. C. E. Bender of Microbiological Associates, Coral Gables, Florida.

Viability of BCG Cultures in Tween-Serum Filtrate Medium

Flask of BCG cultures in the Tween-serum filtrate medium were incubated for 8 days at 37°C. and stored in the refrigerator (approximately 4°C.) for various periods of time (0, 3, or 6 weeks). The number of viable cells (or clumps of cells) in these cultures was determined by spreading 0.15 cc. of 10^{-5} culture dilutions (in 0.1 per cent bovine albumin) on the surface of oleic acid–albumin agar. The number of viable units, computed from the number of colonies detectable after 3 weeks' incubation at 37°C., is reported in Table I.

In a parallel experiment, tubes containing 5 cc. of oleic acid–bovine albumin liquid medium were inoculated with serial dilutions (in 0.1 per cent bovine albumin) of similar BCG cultures stored for 0, 3, or 6 weeks in the refrigerator, as well as of a standard BCG vaccine. This vaccine had been prepared at the Henry Phipps Institute in Philadelphia; it was shipped by Dr. Aronson to our laboratory at the Rockefeller Institute in New York, where it was received the following day and immediately tested. The turbidity of this standard vaccine was approx-

TABLE I

Viability of BCG (Phipps) Culture in Liquid Medium Containing Tween and the Filtrate of Heated Human Serum

Preparation	Incubation time 37°C.	Storage (ice box)	Colonial count* (per cc. of culture)
	days		
I	8	0	12×10^6
II	8	0	33×10^5
II	8	3 wks.	40×10^6
III	8	0	13×10^6
III	8	6 wks.	36×10^6

* Average of counts made on 5 plates of oleic acid–albumin agar. Colonial counts do not describe exactly the number of viable cells as the bacterial suspensions contained many microscopic clumps in addition to single cells.

The differences between the different counts are within the experimental error.

imately half that of the cultures in Tween-serum filtrate medium. On microscopic examination, it was found to consist of a mixture of single cells and of clumps of various sizes in proportion not unlike those present in the Tween cultures. The bacilli in the standard vaccine were uniformly acid-fast and appeared longer than those in the Tween cultures.

The tubes of oleic acid–bovine albumin medium inoculated with graded dilutions of these different preparations were incubated at 37°C. The extent of growth after 14 days' incubation is recorded in Table II.

As seen from the results presented in Tables I and II, growth could be initiated in liquid medium and colonies developed on oleic acid–albumin agar, following inoculation with extremely high dilutions of fresh and stored cultures in Tween-serum filtrate medium. It is clear therefore that a large percentage of the cells present in these cultures were living and physiologically active and that no detectable decrease in their viability occurred during the 6 weeks' storage at 4°C. It will be noted also that the standard BCG vaccine behaved as if it

contained far fewer viable cells than the Tween cultures (less than one hundredth as many). This finding, duplicated with samples of vaccine obtained from other sources, suggests that the preparations of standard vaccine contain a very large percentage of dead cells unless one assumes that these cells are viable but are unable to grow on the albumin media.

TABLE II

Growth in Oleic Acid–Bovine Albumin Liquid Medium of Graded Inocula of Different Preparations of BCG

Inoculum	Standard BCG vaccine	BCG cultures in Tween-serum filtrate medium		
		Fresh culture	Culture stored 3 wks.	Culture stored 6 wks.
cc.				
0.5×10^{-2}	+++	++++	++++	++++
0.5×10^{-3}	+++	++++	++++	++++
0.5×10^{-4}	++	+++	+++	++++
0.5×10^{-5}	−	++	+++	+++
0.5×10^{-6}	−	+	++	++

TABLE III

Effect of Glucose on the Growth and Viability of BCG in Tween-Serum Filtrate Medium

Glucose concentration	No. of viable units* per cc. of culture after the following periods of incubation			
	1 wk.	2 wks.	3 wks.	4 wks.
per cent				
0	17×10^6	36×10^6	31×10^6	19×10^6
0.1	28×10^6	54×10^6	43×10^6	33×10^6
0.5	39×10^6	49×10^6	83×10^5	60×10^2

* Calculated from number of colonies detectable after agar plates had been incubated for 3 weeks at 37°C.

As mentioned in the text, the Tween-serum filtrate cultures consisted in large part of microscopic clumps. The number of living cells was therefore much larger than the numbers of colonies growing on agar.

Effect of Glucose on the Abundance and Viability of Growth of BCG in Liquid Tween-Serum Filtrate Media.—Glucose or glycerine is not essential to secure rapid multiplication of tubercle bacilli in the Tween-albumin medium. It is known on the other hand that these substances can increase markedly the yield of growth in this medium—as measured by dry weight of the bacterial cells or turbidity of the cultures. The purpose of the following experiment was to determine the effect of glucose on the number of viable cells present in BCG cultures after various periods of incubation.

Glucose was added aseptically in 0.1 to 0.5 per cent final concentration to a number of flasks containing 30 cc. of Tween-serum filtrate medium prepared as described earlier in this report. The stock glucose solution (50 per cent) had been autoclaved with 0.01 M citric acid in order to prevent the development of the toxic substances which are formed during heating at neutral or alkaline reactions. The culture flasks were inoculated with 3 cc. of an 8 day old BCG culture grown in the same medium without glucose. Samples of cultures were taken aseptically at weekly intervals and 0.02 cc. of graded dilutions (in 0.1 per cent bovine albumin) was spread on the surface of oleic acid–albumin agar. The number of viable units per cubic centimeter of culture was computed from the number of colonies visible after 3 weeks' incubation at 37°C. (Table III).

The results presented in Table III suggest that addition of glucose to the medium can increase somewhat the yield of bacterial cells. It is true that the differences in numbers of viable units in cultures with and without glucose incubated for 1 and 2 weeks, fall probably within the limits of error of the enumeration technique. Nevertheless, these differences acquire some significance by virtue of the fact that the turbidity of the cultures increased more rapidly with glucose (both in the 0.5 and 0.1 per cent concentration) than without, reaching after 2 weeks in the former case a level approximately twice as high as in the latter.

In contrast with the findings indicating an enhancing effect of glucose on growth, is the observation that an excess of the sugar (0.5 per cent) brought about a marked decrease in the number of viable units on prolonged incubation (3 and 4 weeks). A similar and even more striking decrease in viability occurred when glycerine (0.5 per cent) was added to the medium instead of glucose. Microscopic study revealed that, after 2 to 3 weeks' incubation at 37°C., in the Tween-serum filtrate medium containing 0.5 per cent glucose or glycerine, there were present a large number of bacterial cells that exhibited abnormal morphology and that had lost their acid-fast staining property. These alterations continued to increase on further incubation and finally resulted in extensive disintegration of the cells. No similar change was observed over the same period of time in the flasks to which neither glucose nor glycerine had been added. No analysis has been made of the mechanism of this toxic effect. It appears possible that, under the conditions of submerged growth determined by the presence of Tween in the medium, the rate of oxygen diffusion was not rapid enough to allow complete oxidative utilization of the higher concentrations of glucose or glycerine; and that the tubercle bacilli suffered a biochemical injury resulting from semi-anaerobic metabolism.

As the goal of the present study was the preparation of BCG cultures to be used as experimental living vaccines, viability was deemed a more important criterion than yield of bacterial cells in the selection of the culture medium. For this reason glucose and glycerine were omitted from the Tween-serum filtrate media used in the investigation of the antigenic activity of BCG, to be reported in the following paper.

SUMMARY

Diffuse, submerged growth of BCG bacilli has been obtained in liquid media containing 0.02 per cent Tween 80 and the soluble fraction of human serum heated under acid conditions (pH 2.5) at 65°C.

In the absence of glucose or glycerine,—which had a detrimental effect on viability—these cultures consisted predominantly of cells that were living and that remained viable during prolonged storage at temperatures ranging from 4 to 37°C.

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PRODUCTION OF BCG VACCINE IN A LIQUID MEDIUM CONTAINING TWEEN 80 AND A SOLUBLE FRACTION OF HEATED HUMAN SERUM

II. ANTIGENICITY OF THE CULTURE AFTER VARIOUS PERIODS OF STORAGE

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As shown in the preceding paper, it is possible to obtain submerged, diffuse growth of strains of BCG, by cultivating them for 8 days at 37°C. in a liquid medium containing the wetting agent Tween 80 and the soluble fraction of heated human serum. A high percentage of the organisms present in these cultures can survive prolonged periods of storage at ice box and incubator temperatures (1).

The present paper deals with the antigenic response of guinea pigs to the injection of cultures grown under these conditions and stored at 4°C. for periods as long as 6 weeks.

EXPERIMENTAL

The culture media, the bacteriological techniques, and the strain of BCG used in the present study have been described in the preceding paper (1).

The guinea pigs were female, smooth haired albino animals of the Rockefeller Institute stock. They came from a colony selected by Dr. M. W. Chase (2) on the basis of high susceptibility to sensitization with 2:4 dinitrochlorobenzene. The animals were pen-inbred, only the bucks being selected for susceptibility to sensitization.[1] The stock has been rendered and maintained free of carriers of group C streptococci by skin testing.

Throughout the experiment, the guinea pigs were kept in the same room, which was constantly irradiated with a General Electric germicidal lamp (2537 λ wave length). They were housed in groups of two or three per cage and were fed a diet of hay, oats, and cabbage.

At the beginning of the study, on March 9, 1949, they were tested for sensitivity to tuberculin by the intracutaneous injection of 0.005 mg. of PPD (Sharp & Dohme). None of them gave a positive reaction.

Effect of Dose of BCG on the Development of Tuberculin Allergy.—The purpose of the following experiment was to determine the minimal number of units of BCG capable of rendering guinea pigs allergic to tuberculin.

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[1] We are informed by Dr. M. W. Chase that these animals, although uniform with reference to their susceptibility to sensitization with 2:4 dinitrochlorobenzene, are heterogeneous in their response to sensitization to tuberculin.

Guinea pigs weighing approximately 300 gm. were injected subcutaneously (in the groin) or intracutaneously with various dilutions in 0.1 per cent albumin of a culture of BCG grown in the liquid medium containing Tween 80 and the soluble fraction of heated human serum.

The number of viable units in the culture was determined by plating serial dilutions of

TABLE I

Effect of Injection of Different Doses of Living BCG on the Production of Tuberculin Allergy in Guinea Pigs

No. of viable units of BCG injected	Route of injection	Tuberculin test			
		Material used	Time interval between injection of BCG and tuberculin test	Result at 48 hrs.	
				Diameter of erythema	Remarks
			wks.	*mm.* $\times$ *mm.*	
400,000	Subcutaneous	Old tuberculin (dilution 1/1000)	5	17 $\times$ 15	*Cocarde*
				12 $\times$ 12	Necrotic center
				12 $\times$ 10	—
4,000	"	" "	"	13 $\times$ 12	*Cocarde*
				15 $\times$ 12	Necrotic center
				10 $\times$ 10	" "
40	"	" "	"	15 $\times$ 10	" "
				10 $\times$ 8	—
"0.4"	"	" "	"	0	—
				0	—
1,100,000	Intracutaneous	PPD 0.0005 mg.	6	17 $\times$ 15	*Cocarde*
				14 $\times$ 14	Necrotic center
11	"	" " "	"	11 $\times$ 9	" "
				8 $\times$ 8	—
"1"	"	PPD 0.005 mg.	"	0	—
				0	—

it on oleic acid–bovine albumin agar (3, 4). Five to 6 weeks later, the animals were tested for sensitivity to tuberculin by the injection of either old tuberculin or PPD (Sharp & Dohme) (Table I).

It is apparent from the results presented in Table I that guinea pigs can be sensitized to tuberculin by the injection—either subcutaneous or intracutaneous—of less than 100 viable BCG units. Moreover the tuberculin reactions

elicited by the injection of either O.T. or PPD 6 weeks after vaccination were approximately of the same intensity with all doses of vaccine except with those containing very few viable units.

Local Lesions Produced by BCG.—As will be pointed out in a later section of this report (see Table IV), the intracutaneous injection of 0.05 cc. of BCG cultures grown in the Tween-serum filtrate medium resulted in lesions larger than those produced by the intracutaneous injection of 0.05 cc. of a BCG vaccine prepared by the standard technique (5). An experiment was instituted to test whether this difference was due to a greater intrinsic virulence of the former

TABLE II

Lesions Arising in Guinea Pigs Following the Intracutaneous Injection of Various Doses of Living BCG

Substrain of BCG*	No. of viable units per cc. of undiluted subculture	Guinea pig No.	Lesion arising at sites of injection of various doses of BCG culture						
			Un-diluted	10^1	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
			mm.	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
Culture carried for 2 years in Tween-albumin medium	25×10^6	2-07	10‡ Ulcer	7	4	2	Tiny nodule	Tiny nodule	—
		38-46	10 Ulcer	6 Ulcer	"	"	" "	" "	—
First transfer from sample of standard BCG vaccine	11×10^6	2-07	9 Ulcer	6	3	3	" "	2 (erythema only)	—
		38-46	9 Ulcer	5 Ulcer	4	3	" "	" "	—

* Both substrains subcultured in Tween-serum filtrate medium; the numbers of viable units reported here are within the range of errors of the enumeration technique as used in this experiment.

‡ Diameter in millimeters of the red nodule at the infection site 7 days after injection; examination for ulcers made 14 days after injection.

cultures, or to the fact that they contained more viable organisms than the standard vaccine.

The two substrains of BCG (*i.e.* the one carried for 2 years in our laboratory in Tween-bovine albumin medium, and one supplied for this particular experiment in the form of a sample of standard vaccine by Dr. Aronson) were inoculated into the liquid medium containing Tween and human serum filtrate. After 8 days' incubation at 37°C., 0.05 cc. of serial tenfold dilutions of each subculture was inoculated intracutaneously into two guinea pigs, one series of dilutions on either side. The sizes of the resulting lesions, observed 1 week and 2 weeks after inoculation, are recorded in Table II.

The results presented in Table II show that the culture of the BCG strain maintained for 2 years in the Tween-bovine albumin medium and the culture

recovered from a new sample of standard BCG vaccine, produced lesions of indistinguishable severity when comparable dilutions of growths in ' een-serum-filtrate medium were injected intracutaneously into normal guinea pigs.

Antigenic Response of Guinea Pigs to Vaccination with Various Preparations of BCG.—The following experiment was designed to determine the effect of storage at 4°C. on the antigenic efficacy in guinea pigs of cultures of BCG which had grown diffusely in a liquid medium containing Tween 80 and a filtrate of heated human serum. A BCG vaccine prepared by the conventional technique, and used within 48 hours after its preparation, served as a standard of reference.[2]

Half the guinea pigs used in this experiment were 4 to 5 months old and had weights of the order of 500 gm. at the beginning of the experiment; the others were 2½ to 3 months old and weighed approximately 300 gm. Equal numbers of animals of the two age groups were assigned by random selection to each of the experimental groups. Vaccination with the bacterial suspensions (or uninoculated medium in the control animals) was administered on March 15, 1949. It consisted of two simultaneous intracutaneous inoculations, one over each shoulder, each of 0.05 cc. of the standard vaccine, or undiluted culture, or uninoculated medium.

A tuberculin test was made on April 26, 6 weeks after vaccination, 0.0005 mg. tuberculin PPD (Sharp and Dohme) being used for the vaccinated animals and 0.005 mg. for the controls.

The challenge infection was carried out on May 6th by injecting subcutaneously over the sternum 0.1 cc. of an undiluted culture in Tween-bovine albumin medium, of virulent human tubercle bacilli of the Amerzanga strain;[3] the inoculum contained 13 million viable bacterial units (single cell or small clumps). Some of the guinea pigs which had received the BCG vaccines were kept uninfected to serve as controls on the virulence of the vaccines.

At weekly intervals from the beginning of the experiment, all animals were weighed on a spring balance, weights being read to the nearest 5 gm. They were inspected at the same time to detect the occurrence and measure the extent of the local skin lesions produced by the injections of BCG and, later, of virulent tubercle bacilli. The size of the axillary and inguinal lymph nodes was also estimated by palpation. Necropsies were performed as soon as possible after death on all animals that died during the course of the experiment, and sections of the liver, spleen, and lungs were made and stained with hematoxylin and eosin, and by the Ziehl-Neelsen technique. The experiment was concluded on July 15, approximately 10 weeks after infection with the virulent tubercle bacilli. Ten of the sixteen unvaccinated animals had already died, and the survivors of all groups were killed with chloroform and necropsied during the next 4 days. Sections were made of lesions not obviously tuberculous, as well as of the lungs, liver, and spleen of all animals that had received only BCG vaccine (groups IB and IIB).

The details of the subdivision of guinea pigs into several groups are shown in Table III, and some of the results of the vaccination and challenge infection in Table IV.

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[2] This sample of standard vaccine, prepared at the Henry Phipps Institute in Philadelphia, was kindly supplied by Dr. J. Aronson. It was injected into the animals immediately upon receipt, the day after it had been issued from the Henry Phipps Institute.

[3] This strain was kindly supplied by Dr. J. Aronson.

Thirteen animals died between commencement of the experiment on March 3 and its completion on July 14. Ten of these were non-immunized infected animals (group V), and they all died of tuberculosis, as did No. 3-55 of group IA

TABLE III

Protocol of Experiment on Vaccination of Guinea Pigs with Different Preparations of BCG

Group	No. of animals	BCG injection Mar. 15-16, 1949			Tuberculin test Apr. 26, 1949	Challenge infection May 6, 1949	Survivors sacrificed on
		Strain	History of vaccine	Viable units in dose injected			
IA	16	Standard vaccine	Used within 48 hrs. of preparation of vaccine	13 × 10³	0.0005 mg. PPD	13 × 10⁶ bacilli*	7/15/1949
IB	8	" "	" "	"	"	Sterile medium	7/14/1949
IIA	17	Culture in Tween-heated serum filtrate	Grown for 8 days. No further treatment. No storage	12 × 10⁵	"	13 × 10⁶ bacilli	7/18/1949
IIB	8	" "	" "	"	"	Sterile medium	7/14/1949
III	17	" "	Grown for 8 days. Culture stored for 3 wks. at 4°C.	13 × 10⁵	"	13 × 10⁶ bacilli	7/19/1949
IV	"	" "	Grown for 8 days. Culture stored for 6 wks. at 4°C.	35 × 10⁵	"	13 × 10⁶ bacilli	7/19/1949
V	16	Sterile Tween-serum filtrate		0	0.005 mg. PPD	13 × 10⁶ bacilli	7/14/1949

* Culture of human strain "Amerzanga" grown for 8 days in Tween-bovine albumin medium.

which had received the standard vaccine. The other two fatal cases, No. 3-04 of group IA and No. 3-79 of group IV, died 31 and 40 days after challenge inoculation. The only microscopic evidence of generalized tuberculosis in No. 3-04 was enlargement of the axillary lymph nodes, which on histological examination showed tubercles and early caseation. Sections of the other organs

TABLE IV

Response of Guinea Pig to Vaccination with BCG and to Infection with Virulent Human Tubercle Bacilli

No.*	Weight‡	BCG lesions Diameter on 3/23	Ulceration on 3/30	Spleen* Size	Spleen* Lesions§	Liver Enlargement	Liver Lesions§	Lungs Lesions§	Lymph nodes‖ Tracheobronchial	Lymph nodes‖ Axillary
		mm.		*mm.*						
					Guinea pig group IA*					
3-55	Dead 6/30	0	—	65 × 25	Many necrotic	+++	Many necrotic	Many	+++C	C
2-39	500–580	3	—	50 × 22	" large	+,	Few large	Few	+++C	"
2-64	550–670	5	—	45 × 21	" "	—	" small	"	+	"
2-72	580–740	4	—	42 × 20	" "	—	" "	Many	+++	"
3-73	430–690	3	—	40 × 16	Few small	—	0	0	+	"
3-05	615–715	5	—	40 × 15	Many large	—	Few small	0	++	
3-03	590–700	3	—	36 × 17	Few "	±	" "	Few	+	"
3-54	330–565	3	—	35 × 20	" "	—	" "	"	++	"
2-47	375–610	3	—	35 × 15	" small	—	" "	" small	++	"
3-14	595–750	5	—	35 × 14	" "	—	0		—	"
2-30	560–685	1	—	33 × 17	" large	—	Few small	"	+++	"
3-56	275–550	3	—	33 × 15	" "	—	" "	"	++	"
2-36	340–605	1	—	32 × 15	0	—	0	0	+	"
3-59	330–555	3	—	32 × 13	Few large	—	0	Few	—	"
2-28¶	335–590	2	—	30 × 15	" small	—	Few small	0	+	"
3-04	Dead 6/6	5	—	32 × 13	0	—	0	0	—	"
					Guinea pig group IB*					
3-87	295–610	3	—	35 × 12	None	—	None	None		
2-32	570–710	2	—	34 × 16	"	—	"	"		
3-06	540–725	2	—	34 × 11	"	—	"	"		
3-94	330–675	3	—	30 × 13	"	—	"	"		
3-64	325–625	1	—	30 × 13	"	—	"	"		
3-32	440–600	3	—	30 × 12	"	—	"	"		
2-73	255–560	1	—	30 × 11	" "	—	"	"		
2-70	600–700	2	—	30 × 10	"	—	"	"		

* Animals arranged in order of decreasing spleen size.

‡ The two figures given under "Weight" refer to the weight of the animal at the beginning of the experiment (just before vaccination with BCG) and at the end.

§ The data presented in this table refer only to macroscopic findings. The expression "few" lesions indicates that less than 10 isolated lesions were seen on macroscopic examination. "Many" lesions indicates more than 10.

‖ C = caseation. Only evidence of macroscopic caseation was recorded.

¶ Non-specific death.

TABLE IV—*Continued*

No.*	Weight‡	BCG lesions		Necropsy findings						
		Diameter on 3/23	Ulceration on 3/30	Spleen*		Liver		Lungs	Lymph nodes‖	
				Size	Lesions§	Enlargement	Lesions§	Lesions§	Tracheobronchial	Axillary
	gm.	*mm.*		*mm.*						
					*Guinea pig group IIA**					
3-90	375–465	12	+	57 × 27	Semiconfluent	+	Many large	Many	+++	C
3-18	610–565	"	+	45 × 24	"	+	Many small	Few	+	"
2-56	520–560	"	+	45 × 22	Many large	−	Few small	"	−	"
3-08	570–460	"	+	45 × 22	Confluent "	+	" large necrotic	0	+	"
2-35	665–630	"	+	45 × 22	Many "	++	Many small	Many	+++C	
3-68	355–615	"	−	43 × 19	Few "	+	" "	Few	+++	"
3-67	360–570	"	+	40 × 22	" "	−	" "	"	+++	"
3-20	480–700	"	+	37 × 15	0	−	0	"	+	
3-09	600–705	"	+	35 × 15	Few small	−	Few small	0	+	
3-66	325–570	"	+	35 × 15	" "	−	" large necrotic	Few	−	"
3-17	555–680	14	+	34 × 16	0	−	Few	0	±	"
3-10	620–675	10	+	34 × 13	Few small	−	0	0	+	"
3-62	310–580	12	+	34 × 11	" "	−	0	0	±	
3-12	530–595	"	+	33 × 16	Many large	−	Many small	Few˙	++	"
2-48	260–505	10	+	32 × 13	Few "	±	" "	"	+++	"
3-57	270–525	"	+	32 × 13	" small	−	0	Many	++	"
3-71	320–590	"	+	30 × 15	0	−	Few small	0	+	"
					*Guinea pig group IIB**					
3-70	360–665	12	+	35 × 15	Nonc	−	None	None		
2-50	455–655	"	+	33 × 15	"	−	"	"		
3-49	580–725	"	+	33 × 15	"	−	"	"		
3-97	320–635	"	+	33 × 13	"	−	"	"		
2-44	330–545	"	+	32 × 14	"	−	"	"		
4-00	330–605	"	+	32 × 12	"	−	"	"		
2-43	550–765	15	+	31 × 14	"	−	"	"		
3-48	580–650	12	+	28 × 13	"	−	"	"		

TABLE IV—*Continued*

No.*	Weight‡	BCG lesions		Necropsy findings						
		Diameter on 3/23	Ulceration on 3/30	Spleen*		Liver		Lungs	Lymph nodes	
				Size	Lesions	Enlargement	Lesions§	Lesions§	Tracheo-bronchial	Axillary
	gm.	*mm.*		*mm.*						
2-52	600–535	14	+	54 × 24	Semiconfluent large	++	Many large necrotic	Many	++++C	C
3-28	585–660	10	+	54 × 22	Many large	−	0	"	+++	"
3-16	680–625	"	−	50 × 25	Semiconfluent large	++	Many small	"	++++C	"
2-26	330 × 560	8	+	45 × 21	Few large	−	Few "	"	++++	"
2-41	595 × 670	10	+	45 × 20	Many "	+	" "	"	++	"
2-49	230 × 465	8	+	45 × 18	Few necrotic	−	" large	Few	+++	"
3-77	300–560	10	+	42 × 20	Many large	−	"	Many	++	"
2-42	260–520	6	+	39 × 22	Confluent	−	" necrotic	"	++++	"
3-75	300–480	8	+	37 × 22	Few large	−	Few small	Few	+++C	"
3-46	490–635	10	+	37 × 17	" "	+	Many small	Many	+++	"
3-29	650–675	"	+	35 × 14	Many small	+	" "	Few	++	
2-38	640–670	12	+	35 × 12	" "	−	0	0	+	"
3-22	520–590	14	+	33 × 17	" "	−	0	Few	±	"
3-74	370–625	10	+	33 × 13	" "	−	Few small	0	++	"
3-13	560–625	12	+	30 × 12	" "	−	" "	Few	±	"
3-72	325–565	10	+	27 × 13	" "	−	0	0	±	"
2-31	265–470	"	+	35 × 17	" large	−	Few small	Many	+++C	

Guinea pig group III*

TABLE IV—*Continued*

No.[*]	Weight[‡]	BCG lesions		Necropsy findings						
		Di-ame-ter on 3/23	Ul-cera-tion on 3/30	Spleen[*]		Liver		Lungs	Lymph nodes	
				Size	Lesions	Enlarge-ment	Lesions[§]	Le-sions[§]	Tracheo-bronchial	Axil-lary
Guinea pig group IV[*]										
	gm.	*mm.*		*mm.*						
3-91	350–405	8	+	74 × 30	Semiconfluent necrotic	++	Many large necrotic	Many	++	
3-39	530–505	10	+	58 × 25	" "	++	" "	"	++++	C
3-92	340–535	8	+	55 × 25	Confluent large	—	Few small	Few	++	"
3-81	285–490	"	+	50 × 25	" "	+	Many "	Many	+++	"
3-85	210–530	"	+	43 × 18	Many large	+	" "	Few	+	"
3-33	575–645	10	+	40 × 15	" "	+	Many small	Many	++++C	"
2-29	595–635	"	+	38 × 18	" "	—	Few small	"	+++	"
3-78	305–605	"	+	37 × 16	" "	—	0	0	+	
2-33	555–650	"	+	35 × 20	" "	—	Few small	Many	++++C	"
2-46	640–790	"	+	35 × 17	Few small	—	0	Few	++	
2-57	555–670	"	+	35 × 15	" "	—	Few small	0	++	"
3-26	675–700	"	+	35 × 15	" large	—	0	Few	++	"
3-52	475–800	"	+	35 × 13	0	—	0	0	±	
3-80	335–640	6	+	33 × 15	Few small	—	0	0	+	"
3-86	270–635	6	+	32 × 14	" "	—	0	Few	+	"
3-31	645–665	10	+	30 × 15	" "	—	Few small	"	+++	"
3-79[¶]	Dead 6/15	8	+	30 × 12	" "	—	0	0	—	"

TABLE IV—*Concluded*

No.*	Weight‡	Spleen* Size	Spleen* Lesions	Liver Enlargement	Liver Lesions§	Lungs Lesions§	Lymph nodes Tracheo-bronchial	Lymph nodes Axillary
		mm.						

Guinea pig group V*

No.*	Weight‡	Size	Lesions	Enlargement	Lesions§	Lesions§	Tracheo-bronchial	Axillary
	gm.	*mm.*						
3-88	Dead 7/13	90 × 28	Many necrotic	++	Semiconfluent necrotic	Many	+++C	C
3-96	" 7/4	75 × 25	Semiconfluent necrotic	+++	Confluent necrotic	"	±	"
3-82	305–590	73 × 34	Confluent necrotic	+++	" "	"	+++	"
3-35	Dead 7/8	55 × 24	Many necrotic	+++	Semiconfluent necrotic	"	++C	"
3-50	" 7/14	53 × 30	Semiconfluent necrotic	+++	" "	"	+++C	"
3-34	550–630	53 × 30	Semiconfluent	++	Many small	"	++++C	"
3-43	530–600	53 × 25	Many small	++	" "	Semi-con-fluent	+++	"
3-89	Dead 6/9	50 × 27	Semiconfluent necrotic	+++	Semiconfluent necrotic	Many	++C	"
2-61	" 6/27	50 × 24	Many necrotic	+++	Many necrotic	"	+++C	"
3-83	" 6/22	50 × 20	" "	+++	Semiconfluent necrotic	"	+++C	"
2-34	680–750	47 × 25	Semiconfluent necrotic	++	Many necrotic	"	+++	"
2-27	365–550	47 × 24	Many necrotic	++	Many small	"	+++	"
3-36	Dead 6/25	45 × 21	" "	+++	" necrotic	"	+++	"
3-84	" 6/14	45 × 19	" "	+	Few "	Few	+++C	"
3-93	300–610	43 × 23	Semiconfluent necrotic	+++	Many "	Many	+++	"
3-40	Dead 7/14	40 × 20	Few small	+++	Semiconfluent necrotic	Semi-con-fluent	+++C	"

failed to reveal any abnormality. This animal may have died as a result of injury, for the peritoneal sac contained a considerable quantity of bloody fluid. The axillary and inguinal lymph nodes of No. 3–79 were enlarged and caseous, and there were a few necrotic foci in the spleen but no other macroscopic or microscopic evidence of generalized tuberculosis. The cause of death was not determined.

The results of the various procedures carried out in the course of the experiment can be briefly summarized as follows:—

In all animals which had received the standard BCG vaccine (groups IA and IB) the slight local ulcer observed at the site of vaccination had healed by March 23, 1 week after the injection of the vaccine. At this time there were left only small hard red lumps between 3 and 5 mm. in diameter. These persisted until April 6 and all had completely disappeared by April 20. All animals which had received the fresh BCG culture grown in Tween-serum filtrate medium (groups IIA and IIB) exhibited lesions exceeding 10 mm. in diameter and reaching up to 15 mm. The lesions were in general slightly smaller in animals vaccinated with the culture stored for 3 or 6 weeks (groups III and IV). All animals of groups IIA, IIB, III, and IV (i.e. those receiving the Tween cultures fresh or stored) exhibited on March 30 an ulcer which was found to be healed on April 13 (in only one animal of group IIB did the ulcer remain open until April 20). It is clear therefore that the local lesions developing from the intracutaneous injection of BCG were considerably larger, and persisted much longer, in the animals receiving the cultures grown in the medium containing Tween and filtrate of heated serum than in the animals receiving the standard vaccine. It may be inferred from the results of the experiment described earlier in this paper, in which two guinea pigs were inoculated with various doses of subcultures, in the same fluid medium, of the two substrains of BCG that this difference was due principally to the larger number of viable organisms in the Tween-serum filtrate cultures.

All vaccinated animals exhibited intense tuberculin allergy when tested on April 26; while the control animals (group V) failed to react to a 0.005 mg. PPD. The severity of the tuberculin reaction was almost identical in all vaccinated groups, whether it was measured in terms of the average diameter of the erythematous area (varying from 10 × 12 mm. to 20 × 20 mm.), or by the proportion of animals which showed the "cocarde" or triple response, consisting of a livid center, an inner white zone, and an outer erythematous area.

A large mass, at first hard but later becoming fluctuant, developed at the site of the challenge infection in all animals. Ulceration of this abscess occurred much earlier in the vaccinated than in the non-vaccinated animals (Table V); amongst the vaccinated guinea pigs those of group IV, which had received the BCG culture stored for 6 weeks in the refrigerator, developed ulcers somewhat later than those of the other groups.

The response of the guinea pigs to inoculation with virulent tubercle bacilli is given in Table IV. The animals within each group are tabulated in order of decreasing spleen size as this was considered the most objective single index of the intensity of the infection. At the conclusion of the experiment all the unvaccinated animals had either died of tuberculosis or showed severe tuber-

culous lesions of the liver, spleen, and lungs. All animals which had received BCG but no challenge infection were gaining weight and in none of them were tuberculous lesions found by macroscopic or microscopic examination. Among the vaccinated animals the only death from tuberculosis occurred in group IA,[4] which had received the standard vaccine, but many other animals of this group appeared to be less severely diseased than animals of the other vaccinated groups. It is obvious that the severity of the disease varied so much from one animal to the other within each group and that there was so much overlapping from one vaccinated group to the other, that the differences observed had no significance. It is of particular interest to point out that no significant difference could be detected between the animals of groups IIA, III, and IV. In each group there were two or three guinea pigs that were losing weight and that had extensive lesions of the spleen and lungs, and moderately severe lesions of the

TABLE V

Date of Ulceration of the Abscess Produced at the Site of Injection (on May 6th) of Virulent Tubercle Bacilli

Group of guinea pigs	No. of animals	No. of animals showing ulceration of subcutaneous abscesses for the first time on the following dates:										
		5/11	5/18	5/25	6/1	6/8	6/15	6/22	6/29	7/6	7/13	No ulceration
IA	16	7	5	1	2	1						
IIA	17	4	10	3	0	0						
III	16	1	9	4	2	0						
IV	17	2	2	6	4	0	2	0	0	0	1	
V	16	0	1	3	6	2	0	1	1	0	0	2

liver. The balance of the animals in each group showed lesions of varied but minor severity, some exhibiting only three or four nodules in a spleen of normal size with no macroscopic lesions in the other internal organs.

DISCUSSION

Some of the findings described in the present paper provide information concerning the lack of pathogenicity of the BCG cultures grown in the medium containing Tween and the soluble fraction of heated human serum. It is true that the intracutaneous injection of 0.05 cc. of culture grown in Tween-serum

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[4] It is worth noting that guinea pig 3-55 of group IA which died of tuberculosis had a negligible reaction at the site of injection of BCG (see table IV). Nevertheless this animal reacted strongly to the tuberculin test, the tuberculin reaction measuring 12 × 10 mm. and exhibiting a white center.

filtrate medium resulted in the appearance of local lesions more severe than those produced by 0.05 cc. of standard vaccine. In order to evaluate the significance of this difference however, it must be kept in mind that the numbers of viable bacterial units (determined by plate counts) were much greater in the Tween culture (approximately 10^7 per cc.) than in the standard vaccine (10^5 per cc.). When the two BCG substrains were cultured in the same medium it was found that the intracutaneous injection into normal guinea pigs of doses containing similar numbers of viable units, produced lesions indistinguishable in size and severity. This finding strongly suggests that the bacterial cells which had grown diffusely in Tween-serum filtrate medium had retained the same intrinsic degree of "attenuation" as those present in the vaccine prepared by the standard technique. It illustrates furthermore the fallacy of expressing in terms of units of volume or weight the doses of a living vaccinating agent in the absence of knowledge of its viability or physiological activity.

Although many more living organisms (approximately 100 times as many) were present in the Tween-serum filtrate cultures than in the standard vaccine, and although the former preparations produced larger lesions than the latter at the site of the intracutaneous injection, the intensity of tuberculin sensitization induced was essentially the same in the four groups of animals vaccinated with the four different preparations mentioned in Table III. Here again, experiments carried out with known numbers of viable bacterial units provide a clue for the analysis of these findings. The data presented in Table I reveal that a definite degree of sensitization could be achieved by the injection of doses of BCG culture containing fewer than 100 viable bacterial units. Moreover the intensity of the sensitivity to tuberculin, measured 6 weeks after vaccination, reached an apparent maximum with a vaccinating dose containing 4000 viable units; even an increase many hundredfold in the number of viable units injected for vaccination failed to bring about a detectable increase in the intensity of the sensitivity to tuberculin. It is clear therefore that the design of the experiment outlined in Table III did not permit a comparison of the efficacy of the different preparations used as vaccines, since the numbers of viable units in all these preparations were far greater than the minimal number required for inducing the maximum level of tuberculin allergy detectable by the technique employed.

It is generally accepted that vaccination with BCG results in a definite degree of multiplication of the bacilli *in vivo* and it is probably for this reason that the degree of tuberculin allergy was relatively independent of the vaccinating dose in the experiment under consideration. Freund has established that amounts of heat-killed tubercle bacilli of the order of 0.1 to 0.003 mg. resuspended in mineral oil are required in order to induce an appreciable degree of tuberculin allergy in guinea pigs; the same dose of heat-killed bacilli in aqueous media

would fail to sensitize (6).[5] The amount of bacillary material represented by these weights is many millions of times larger than the weight of living cells of BCG (in aqueous media) which was found adequate to produce maximal sensitization in the present experiment. It appears almost certain, therefore, that the degrees of allergy recorded in the present paper could not be attributed to the mass of bacillary material present in the vaccinating dose but reflect rather an extensive multiplication of the bacilli *in vivo*.

One may assume also that the protection against infection induced in the guinea pigs of groups IA, IIA, III, and IV (Table IV) was the outcome of the multiplication of the vaccinating inoculum *in vivo*. No statistically significant difference could be recognized in the degrees of resistance exhibited by the different groups of vaccinated animals. All had caseous lesions at the site of inoculation (although the ulcer healed rapidly in some of them), and all had enlarged axillary lymph nodes which were usually caseous. In all groups a few guinea pigs exhibited severe tuberculous lesions of the liver, spleen, and lungs while the lesions in most other individuals were confined to a few large nodules in the spleen and small foci in the lungs and liver. Each group also contained some animals in which the only macroscopic lesions of the internal organs were a few foci in the spleen, which itself was not enlarged.

It must be emphasized, however, that differences in the resistance to infection may have existed but could not be brought out under the conditions of the experiment. Indeed, the technique used in the challenge infection test (subcutaneous infection) was not adequate for the measurement and analysis of resistance to infection as it did not lend itself to a clear differentiation between allergy and antibacterial immunity. The allergic reaction elicited by the introduction of the virulent bacilli probably caused a walling off, or even a shedding, of a significant percentage of the infective inoculum thereby decreasing the effective challenge dose. It is possible, indeed likely, that marked differences in the degree of resistance to infection in the four groups of vaccinated animals could have been brought about by changing certain of the possible variables in the experiment:—the amount of vaccine used, the size of the challenge dose, the time elapsed between vaccination and challenge infection, the route of infection, etc.

The experimental results described in this and the preceding paper do not allow therefore a comparison of the antigenic efficacy—either as sensitizing or as immunizing agents—of the four different BCG preparations used for vaccination. They permit one only to state that the culture grown in the Tween-serum filtrate medium compares well qualitatively with the vaccines prepared by the conventional technique and from the quantitative point of view, may even present definite advantages over the latter. The content in viable cells of

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[5] Dr. Freund informs us that a limited, but definite degree of tuberculin allergy can be induced in some guinea pigs by amounts of heat-killed bacilli of the order of 0.001 mg. and even one-tenth this amount resuspended in oil.

the Tween-serum filtrate culture is extremely high as shown by colony counts and by the ability of minute amounts of culture to sensitize guinea pigs to tuberculin. Moreover, the number of viable cells is predictable and remains remarkably constant even after prolonged periods of incubation or of storage. This stability should greatly facilitate the planning of vaccination experiments and the analysis of their results.

Other minor advantages of the dispersed cultures deserve perhaps some brief mention, namely the rapidity with which they can be obtained, the fact that they require for their preparation only standard bacteriological techniques, the elimination of the transfer operations and of the necessity of grinding the bacterial growth before distribution of the final product, their stability in the very medium in which they are grown, which allows ample time for the performance of the control tests required of biological products. Nevertheless, it is obvious that the technique of cultivation of BCG described in these reports should be regarded at present only as experimental procedure; it constitutes merely a demonstration that, by adequate cultural methods, one can obtain suspensions of BCG organisms exhibiting stable physiological and antigenic activity.

SUMMARY

Groups of guinea pigs were vaccinated by the intracutaneous route with cultures of BCG grown in a liquid medium containing Tween 80 and the soluble fraction of heated human serum. After the cultures had been stored at 4°C. for various periods of time, the antigenic response was compared with that of another group of guinea pigs receiving standard BCG vaccine prepared by the conventional technique.

The local lesions occurring at the site of injection of cultures in Tween-serum filtrate medium were more severe than those produced by the standard vaccine. It was shown that this difference was probably due to the much larger number of viable bacilli in the former preparations. A marked degree of sensitization could be produced with culture dilutions containing as few as 10 viable units (single bacilli or small clumps). Slightly larger doses of BCG led to the highest degree of tuberculin allergy detectable by the technique employed. Further increases in the dose of vaccine failed to alter the level of sensibility when the animals were tested with tuberculin 5 weeks after vaccination.

The same degree of sensitization was achieved by vaccination with 0.1 cc. of either the standard vaccine or any of the fresh or stored cultures in Tween-serum filtrate medium. It was shown that these doses contained numbers of living bacilli far greater than the minimal number required to induce maximal sensitization.

Under the conditions used, the guinea pigs vaccinated with cultures of BCG (fresh or stored) grown in the Tween-serum filtrate medium exhibited a marked degree of resistance to subcutaneous infection with virulent tubercle bacilli.

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THE EFFECT OF INJURY BY TOXIC AGENTS UPON OSMOTIC PRESSURE MAINTAINED BY CELLS OF LIVER AND OF KIDNEY*

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In a preceding publication (1) the significance of osmotic changes in the particulate bodies, in large part mitochondria, which occupy a considerable portion of the cytoplasm of cells of liver and of kidney was discussed and experiments to determine the relation of these changes to hydropic swelling of the same bodies caused by injurious agents, namely, chloroform and butter yellow (dimethylaminoazobenzene), were described. In the same and a later publication (2) swelling of mitochondria and removal of reacting mitochondrial material by hypotonic and even by isotonic solutions, *e.g.* Ringer's solution, were described and experiments defining the relation of these histological changes to the specific gravity of the tissue were recorded.

The environment of tissue cells is the fluid in the tissue spaces and this in turn varies with changes in the circulating medium of the body; that is, the blood plasma. It has been established long ago that the osmotic pressure of the blood is maintained at levels which vary within very narrow limits among different mammalian species and within even smaller limits in the same species under changing conditions. The integrity of the red blood corpuscles is dependent upon the maintenance of osmotic homeostasis (3) and they have an osmotic pressure equivalent to that of the plasma, which approximates a 0.15 molar solution of sodium chloride. It has been tacitly assumed that the same holds true for the cells of all fixed tissues. But study of the movement of water in tissues removed from the body and immersed in various fluids has lately shown (4) that solutions of sodium chloride isotonic for parenchymatous cells of liver have twice the molar concentration of sodium chloride present in the blood serum and isotonic with red blood corpuscles. The metabolism of these cells presumably maintains within their cytoplasm a molar concentration which is considerably greater than that of blood serum and of the fluid surrounding them. The following experiments are described because they show that certain injurious agents administered to animals disturb only temporarily the osmotic equilibrium of liver and kidney cells and lower their level of isotonicity so that it approximates for a time that of blood serum and of erythrocytes.

Methods

The initial change of weight of tissue slices immersed in solutions of sodium chloride of various concentrations has been used to determine the concentration that is in osmotic equilibrium with the tissue. Weight of the slices has been measured by a torsion balance and the procedure followed has been that described in an earlier publication (4). The movement of water to or from normal liver tissue during the first 10 minutes of immersion is proportional to the concentration of the immersion fluid and has a linear course when plotted in this relation. The point at which the line crosses the abscissa (Fig. 1) determines the concentration of sodium chloride which has osmotic pressure equal to that of the tissue under examination (Table I). The use of osmotically equivalent concentrations of sodium chloride as a measure of osmotic pressure has been discussed by Lipson and Visscher (3).

Injury by Chloroform

Chloroform selects for injury the hepatic cells about the central veins of the liver and when administered in sufficient quantity by inhalation, by introduction into the stomach, or by subcutaneous injection causes necrosis which with increasing severity may destroy all parenchymatous cells save those immediately about the portal spaces.

The necrotic cells lose their nuclei and their cytoplasm has a homogeneous appearance when stained with acid dyes. These changes first appear after an interval of 6 to 10 hours (5) and are usually assumed to be the result of autolysis. At the periphery of the nectrotic foci surrounding the central veins or immediately about these veins, when the poison in small quantity has failed to cause necrosis, liver cells are swollen, have sharply defined rounded outlines, and contain mitochondria of which a considerable part have lost their characteristic reactions to stains and have become enlarged and vesicular (1). These swollen bodies may give the cytoplasm a vacuolated or even foam-like appearance. Though stains for fat show that these spaces contain none, fat droplets in abundance are usually found in liver cells in a broad zone about the swollen cells.

The progress of repair which follows when chloroform poisoning does not cause death of the animal was described by Whipple and Sperry (5). Granulocytes and mononuclear phagocytes in large number enter the areas of necrosis and after 3 or 4 days the acidophile cytoplasm of necrotic cells has disappeared. Mitotic figures are seen in adjacent liver cells 24 to 36 hours after the administration of chloroform and are numerous after 48 hours. The rapidity with which recovery occurs evidently varies with the extent of necrosis about the central veins.

Chloroform mixed with twice its volume of paraffin oil has been injected into the subcutaneous tissue of the flank of white rats weighing with few exceptions from 150 to 250 gm. and of no selected strain. The quantity has been 0.25 cc. per hundred gm. of body weight. With favorable diet some of the animals that receive this quantity survive (6). Especially noteworthy in relation to the present study is the occurrence of nephrosis characterized by necrosis of some of the cells of the convoluted tubules and the presence of fat droplets elsewhere in the convoluted tubules and in the loops of Henle. When death occurs after 3 or 4 days there is pleural effusion with compression of the lungs and less advanced peritoneal effusion, occasionally with edema about the pancreas.
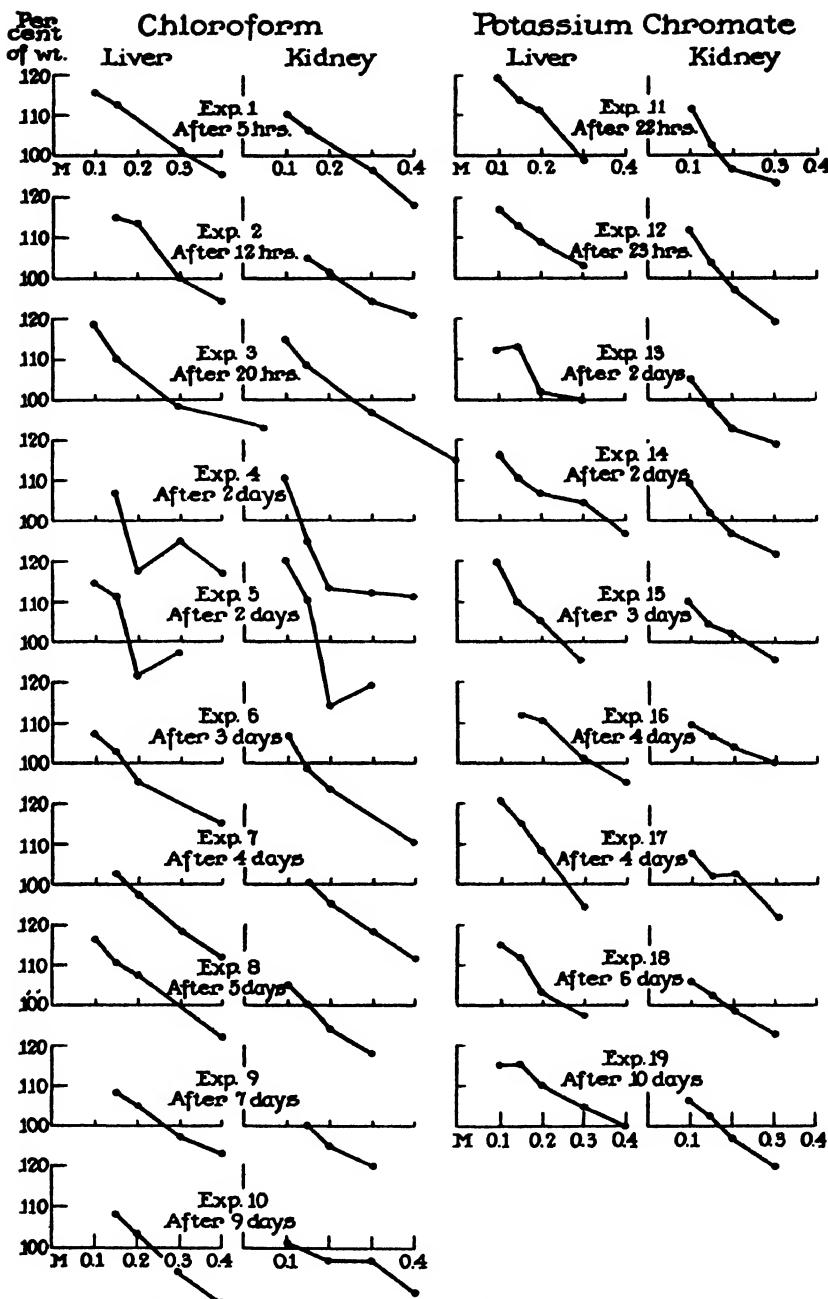
FIG. 1. Changes in the per cent of weight of slices of liver and of kidney tissue found 10 minutes after immersion in solutions of sodium chloride. The tissues were obtained from animals at different intervals after administration (*a*) of chloroform and (*b*) of potassium chromate.

During an interval of 6 to 12 hours immediately following the administration of chloroform, at a time when scant, if any, microscopic change was found, liver tissue maintained its usual isotonicity (Fig. 1, Table I) with solutions of sodium chloride having a concentration somewhat more than twice that in blood serum (4). Of tissues from two animals examined after 20 hours the liver of one had undergone in this relation no significant change but that of the other was isotonic with salt solution of much lower concentration (0.18 molar). After 2 days, at a time when the liver was the site of well defined necrosis, swelling of mitochondria, and deposition of fat about central and hepatic veins, the level

TABLE I

The Effect of Chloroform on the Level of Isotonicity Maintained by Liver Tissue and by Kidney Cortex

Interval after administration of chloroform	Liver		Kidney	
	Molar concentration of sodium chloride isotonic with liver	Maximum intake of water in per cent of weight of liver	Molar concentration of sodium chloride isotonic with kidney cortex	Maximum intake of water in per cent of weight of kidney cortex
Normal average	0.34	200.4	0.25	187.0
5 hrs.	0.32	176.2	0.24	178.0
12 "	0.3	171.5	0.22	169.0
20 "	0.28	178.9	0.27	165.0
20 "	0.18	174.3		
2 days	0.17	149.2	0.14	137.5
2 "	0.18	151.2	0.17	129.6
3 "	0.17	135.2	0.14	118.6
4 "	0.18	136.7	0.16	112.2
5 "	0.3	189.1	0.16	143.7
7 "	0.27	190.5	0.15	138.8
9 "	0.24	177.1	0.17	174.2

of isotonicity was that of 0.17 to 0.18 molar sodium chloride and little above the isotonicity of red blood corpuscles. The same level was maintained during 3 days but after 5 days the liver tissue had approximately regained its usual osmotic relation to solutions of sodium chloride. Concurrently with this change regeneration was in progress and liver cells had almost completely regained their normal appearance. After 9 days (see Table I) the level of isotonicity was below that found in normal animals and with associated nephrosis the liver cells were the site of fat deposition.

In association with severe injury to the cells of the convoluted tubules of the kidney caused by chloroform changes in the osmotic pressure of this tissue were similar to those of the liver. Slices of the cortex less than 1 mm. in thickness and cut parallel with surface of the organ were immersed in various solutions.

As with the liver little morphological change was found with kidney during the early period following chloroform administration but later advanced injury was evident. There was widespread necrosis of the cells of the convoluted tubules with loss of nuclei and disintegration of cytoplasm. Minute globules of fat were found in cells that were not destroyed and tubular casts were usually abundant. Evidence of injury persisted longer in the kidney than in the liver and after 9 days the organ was swollen and pale yellow and casts were numerous within the tubules of cortex and medulla.

During the 1st day following chloroform administration kidney cortex maintained its usual isotonicity with solutions of sodium chloride slightly less concentrated than those isotonic for liver but coincident with the decrease of the level of isotonicity of liver tissue evident on the 2nd day similar change was found in kidney cortex (Fig. 1, Table I) so that immersed slices were in water equilibrium with solutions of sodium chloride from 0.14 to 0.17 molar; that is, the approximate concentration of sodium chloride in blood serum. With the quantity of chloroform that was administered this low level of isotonicity was maintained throughout the experiments and corresponded with histological evidence of severe nephrosis in all the animals.

Immersion of tissues in distilled water has no resemblance to conditions present during life and promptly causes destruction of the tissue. Nevertheless it may give evidence that factors related to osmotic movement of water within the tissue have been altered. Coincident with the fall in the level of isotonicity of liver and kidney tissue caused by chloroform, water intake of the same tissue immersed in distilled water was greatly modified. The tissue took up less water as determined by increase of its weight than normal tissue under the same conditions and a maximum was reached sooner. Fig. 2 shows the effects of chloroform administration on changes in the weight of liver and kidney tissue during 2 hours of immersion in water. 5 hours after chloroform administration weight of the tissue slices, like that of the normal tissues (see Figs. 1 and 8 of an earlier publication (4) increased approximately 80 per cent and reached a maximum after 1 or 2 hours. 20 hours after chloroform administration water intake of liver was little changed but that of kidney was somewhat less than before. After 2 days each took up much less water, reached a maximum sooner, and then rapidly fell. After 2, 3, and 4 days these changes persisted and were more conspicuous with kidney than with liver. After a longer period parallel with histological evidence of repair of liver tissue the graph for liver assumed its original form but the kidney failed to take up water in quantity approximating that of normal tissue until 9 days after chloroform administration (Table I).

Injury by Carbon Tetrachloride

Carbon tetrachloride, like chloroform, injures liver cells and causes greatest change in those about the central veins but injury to the kidney is manifestly less than that with chloroform.

Cameron and Karunaratne (7) injected 0.1 to 0.25 cc. of undiluted carbon tetrachloride into the subcutaneous tissue of white rats. Little change was found after 5 hours but after 24 hours there was necrosis about the central veins and a surrounding zone in which cells had
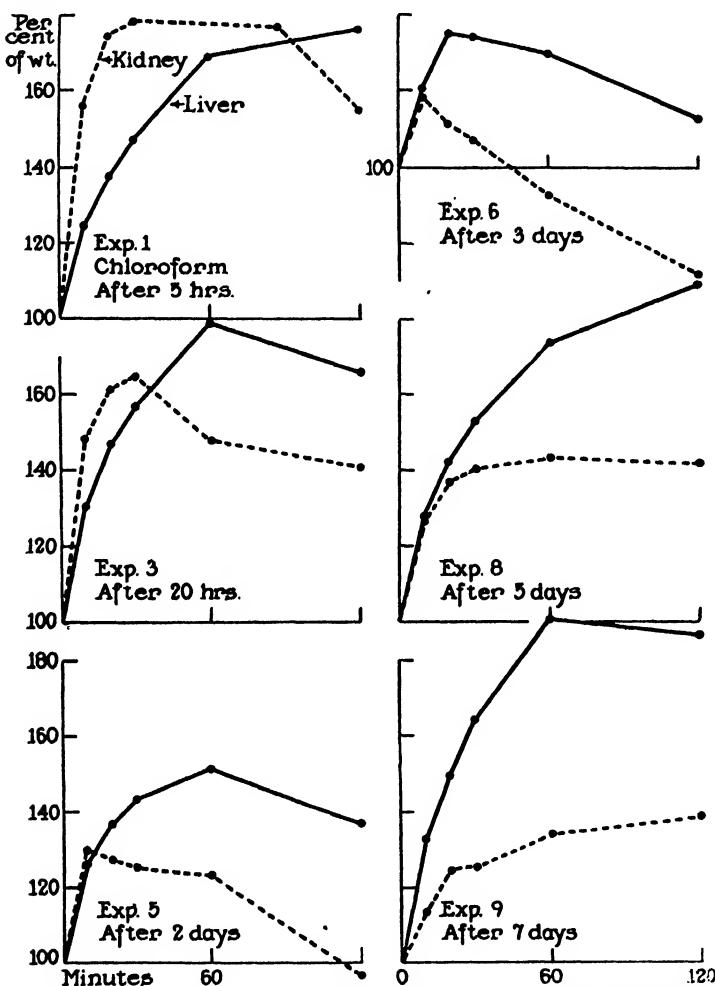
Fig. 2. Changes in per cent of weight of liver slices immersed in distilled water at different intervals after administration of chloroform.

undergone hydropic change. Lymphocytes and histiocytes appeared in the injured tissue. After 3 days necrotic liver cells had disappeared and regeneration characterized by mitotic figures in liver cells was in progress. After 7 days the greater part of the liver appeared to be normal but a few spaces were not filled by regenerated cells.

The movement of water in slices of liver tissue after intervals of 1 to 10 days following the administration of carbon tetrachloride is shown in Table II. The

level of isotonicity fell and after 2 days was that of 0.14 molar sodium chloride, approximating that of blood serum. Later it rose and after 10 days had reached the normal level. Histological changes found in the liver were those that have been cited. In the kidney on the contrary the level of isotonicity underwent

TABLE II

The Effect of Carbon Tetrachloride on the Level of Isotonicity Maintained by Liver Tissue and by Kidney Cortex

Interval after administration of carbon tetrachloride	Liver		Kidney	
	Molar concentration of sodium chloride isotonic with liver	Maximum intake of water in per cent of weight of liver	Molar concentration of sodium chloride isotonic with kidney cortex	Maximum intake of water in per cent of weight of kidney cortex
Normal average	0.34	200.4	0.25	187.0
23 hrs.	0.2	144.3	0.26	186.7+
2 days	0.14	123.1	0.22	173.2
3 "	0.2	160.3	0.26	164.3+
5 "	0.29	167.1	0.25	168.6
10 "	0.34	161.3	0.35	157.7

TABLE III

The Effect of Potassium Chromate on the Level of Isotonicity Maintained by Liver Tissue and by Kidney Cortex

Interval after administration of potassium chromate	Liver		Kidney	
	Molar concentration of sodium chloride isotonic with liver	Maximum intake of water in per cent of weight of liver	Molar concentration of sodium chloride isotonic with kidney cortex	Maximum intake of water in per cent of weight of kidney cortex
Normal average	0.34	200.4	0.25	187.0
22 hrs.	0.3	175.4	0.17	143.5
23 "	0.34	199.7	0.18	140.6
2 days	0.3	170.2	0.14	137.8
2 "	0.36	198.7	0.17	164.1
3 "	0.25	183.6	0.22	147.8
4 "	0.32	193.8	0.3	150.0
4 "	0.26	212.6	0.24	145.0
6 "	0.26	181.5	0.18	164.0
10 "	0.4	164.0	0.17	176.3

little change, falling slightly after 2 days, and no histological evidence of injury was found. The maximum intake of water after immersion of liver in distilled water (see Table III) diminished when the level of isotonicity fell.

Injury by Potassium Chromate

The effect of potassium chromate upon the level of isotonicity of liver or kidney as determined by immersion in solutions of sodium chloride was studied

because this substance causes necrosis of the cells of the convoluted tubules of the kidney cortex, whereas in contrast to chloroform and carbon tetrachloride it leaves the liver apparently uninjured.

The quantity of potassium chromate injected into the subcutaneous tissue was 0.1 cc. of a 2.5 per cent solution, that is 25 mg. per 100 gm. of body weight, save in one experiment (after 23 hours) in which 0.15 cc. was injected.

Within 24 hours slices prepared from the cortex of the kidney lost their normal isotonicity with solutions of sodium chloride approximately 0.26 molar (4) and were in osmotic equilibrium with solutions of sodium chloride 0.17 or 0.18 molar (Table III, Fig. 1). Though the isotonicity of the kidney cortex approached that of blood serum and of erythrocytes, that of liver tissue maintained its usual water equilibrium with solutions of sodium chloride with almost twice this concentration. The level of isotonicity of kidney tissue remained low during 2 days. At this time the proximal convoluted tubules of the cortex had in large part undergone necrosis; nuclei were lost, and the cytoplasm of the cells stained homogeneously with acid dyes. Nevertheless distal convoluted tubules and loops of Henle situated within cortical striae and in the subcortical zone were well preserved though deposition of fat droplets indicated that these structures had undergone minor injury. In two experiments 4 days after administration of potassium chromate isotonicity had risen to approximately normal levels represented by 0.3 and 0.24 molar sodium chloride.

In two experiments 6 and 10 days after administration of potassium chromate the level of isotonicity remained low, being that of 0.18 and 0.17 molar solutions of sodium chloride. In one instance there was persisting widespread necrosis of convoluted tubules and in the other nephrosis characterized by dilatation of tubules and deposition of fat within surviving cells of convoluted tubules and of the loops of Henle and some proliferation of interstitial tissue.

Following administration of potassium chromate the level of isotonicity of liver tissue in no instance diminished below that represented by 0.26 molar sodium chloride. It is noteworthy that the maximum intake of water by liver tissue (Table III) was little changed whereas that of kidney was almost uniformly diminished.

RECAPITULATION AND DISCUSSION

Metabolic changes within the cytoplasm of liver and kidney cells presumably maintain its molecular concentration at a level that establishes osmotic equilibrium with solutions of sodium chloride having a concentration approximately twice that of the blood serum. When excised liver or kidney tissue is immersed in blood serum it takes up water (4). The experiments here described show that

severe injury to liver or to kidney by chloroform causes the level of their isotonicity to fall so that it approximates that of blood serum and of erythrocytes. Like the histological changes that follow injury by chloroform this osmotic change is not immediate and becomes evident after an interval of variable length following administration of the toxic agent. The low level of isotonicity is maintained during several days but after 5 days the level returns to that of normal liver concurrently with regeneration of liver cells. Isotonicity of kidney tissue severely injured by chloroform in the quantity administered, undergoes changes similar to those of the liver but histological evidence of persisting injury and the osmotic pressure, show less tendency to return to normal.

Changes in the isotonicity of liver tissue and kidney cortex are evidently referable to the parenchymatous cells and not to the interstitial tissue of these organs. A preceding study has shown that interstitial tissue of the thymus and the connective tissue of the omentum are approximately isotonic with blood serum (4). It is noteworthy that hypotonic solutions cause great swelling of interstitial tissue of thymus or pancreas whereas the much less abundant fibrous tissue of liver and kidney is little changed; parenchymatous cells and the mitochondria of their cytoplasm are swollen (2).

Carbon tetrachloride which, like chloroform, causes necrosis of parenchymatous cells of liver about central veins likewise causes the level of isotonicity of liver tissue to fall to that of blood serum and it later returns to normal as repair is effected. Injury to the kidney is less with carbon tetrachloride than with chloroform and the level of isotonicity of kidney tissue is little changed.

Potassium chromate, which injures severely the cells of the proximal convoluted tubules of the cortex of the kidney reduces the level of isotonicity of kidney cortex approximately to that of blood serum but scarcely changes that of liver, which escapes structural injury. Recovery from this state of diminished isotonicity may occur promptly and after 3 or 4 days isotonicity may have returned to a normal level. In some instances, with persistence of severe injury recognizable by histological examination, its low level may persist.

Immersion of liver tissue in distilled water is a drastic procedure with little resemblance to processes within the living body. Nevertheless it may give evidence that structures upon which osmotic interchange depends have been profoundly modified. The experiments show that fall of the level of isotonicity of liver tissue or of kidney cortex exposed to water is accompanied by diminution of water intake by the tissue, when compared with that of the normal tissue immersed in water; water intake reaches a maximum sooner; and then falls rapidly.

Pancreas has a level of isotonicity somewhat higher than that of liver (4). The osmotic pressure maintained by cells of liver, kidney, pancreas, and perhaps other glandular organs has doubtless a significant relation to the functional

activity of these organs. It is noteworthy that hepatomas derived from liver cells, and cholangiomas, maintain an osmotic pressure (4), which is little greater than that of the fluid and red corpuscles of the blood.

CONCLUSIONS

As shown in a previous paper the cells of the liver and of the kidney maintain an osmotic pressure approximately twice that of blood and of erythrocytes, exceeding this slightly in the case of liver and being slightly less in that of kidney.

When liver cells are injured by chloroform or by carbon tetrachloride the osmotic pressure they maintain falls to the level of the medium that surrounds them but is promptly restored when recovery from the injury, with some regeneration of liver cells, occurs.

When nephrosis is caused by potassium chromate or by chloroform the osmotic pressure maintained by parenchymatous cells of the renal cortex falls to that of the medium about them but returns to its normal level with recovery from the injury.

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A NEW AND CONVENIENT SYNTHESIS OF 4-AMINO-5-IMIDAZOLECARBOXAMIDE

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An amine formed by *Escherichia coli* during sulfonamide bacteriostasis
(1) has been identified as 4-amino-5-imidazolecarboxamide (V) (2). Since
sulfonamide inhibition apparently blocks the biosynthesis of purines, the
suggestion has been made (2) that the aminoimidazole carboxamide (V) is a
precursor of purines and accumulates in the medium due to a blocked reac-
tion involving the insertion of the single carbon atom necessary for the com-
pletion of the purine ring system. Further study of the possible metabolic
rôle of this amine would be facilitated by an adequate source of material.
A synthesis described by Windaus and Langenbeck (3) starting with
methylglyoxal was found to provide an over-all yield of 1 per cent of the
desired base. Therefore, an alternate method of preparation was under-
taken. Making use of a new imidazole synthesis in which a formamido-
acetamidine is cyclized to a 4-aminoimidazole, we have been able to prepare
4-amino-5-imidazolecarboxamide in an over-all yield of 30 per cent, starting
with ethyl cyanoacetate.

The imino ether of ethyl cyanoacetate (I) was treated with alcoholic
ammonia, simultaneous introduction of the amidine and amide groups taking
place. The resultant malonamamidine (II) was coupled with benzenedia-
zonium chloride to yield the phenylazo derivative (III). When the azo
compound was reduced with zinc dust in 98 per cent formic acid, the
formamido derivative of malonamamidine (IV) was obtained. The formyl
group in this compound exhibited the expected lability to acid, undergoing
ready deformylation to the amino amidine (VII). As the free base, the
formamido amidine (IV) hydrolyzed in aqueous solution to formamido-
malonamide (VI).

Ring closure of the formamido amidine (IV) to 4-amino-5-imidazole-
carboxamide (V) was achieved most conveniently merely by melting the
formamido compound as the hydrochloride. Eventually the mass crystal-
lized as amino imidazole hydrochloride. At 170°, for example, the thermal
cyclization was complete in 10 minutes, yielding 89 per cent of V after one
recrystallization. Although the base hydrochloride was described by
Stetten and Fox (1) as having a melting point of 210–215°, the hydro-

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chloride obtained in both the new synthesis and that of Windaus and Langenbeck melted at 255–256°. The free base and picrate, however, had properties in agreement with both the natural material (1) and that produced by the older synthesis (3).
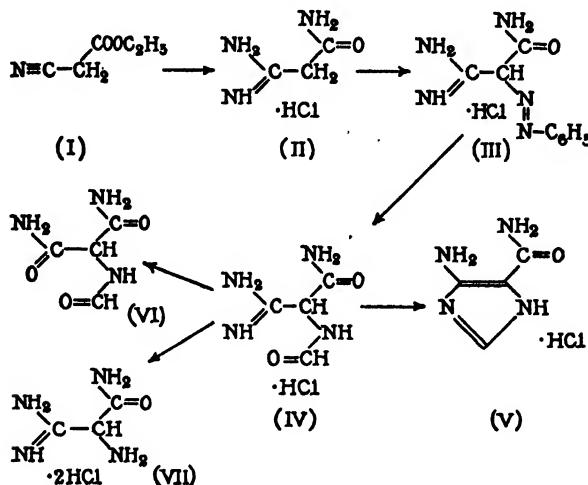
The possibility of achieving new syntheses of purines through intermediate imidazoles made readily accessible by the new ring closure is being investigated.

EXPERIMENTAL

Melting points were determined in a copper block and are uncorrected.

Malonamamidine Hydrochloride (II)—Ethyl cyanoacetate was converted to the imino ethyl ether hydrochloride, m.p. 213–215°, in a 79 per cent yield as described by Glickman and Cope (4). Dried, pulverized imino ether hydrochloride (30 gm.), suspended in absolute alcohol (300 ml.) previously saturated with ammonia, was occasionally shaken while gaseous ammonia was bubbled through for 4 hours. The suspension was kept at room temperature in a stoppered flask for 5 days. Although initially gradual solution of the imino ether was apparent, crystals of amidine hydrochloride soon separated and complete solution was not observed. The product was finally filtered and combined with a second crop obtained on concentration of the filtrate, yielding 15.8 gm., 76 per cent, m.p. 169–175°. For an analytical sample, a solution of the amidine hydrochloride in a minimum amount of water was treated with 5 volumes of absolute alcohol; ether was added until crystallization began. The needles thus produced melted at 176–177°.

$C_3H_8ON_3Cl$. Calculated, Cl⁻ 25.80, N 30.54; found, Cl⁻ 25.64, N 30.61

Pinner mentioned the synthesis of this amidine (5) without giving details and recorded the melting point as 150°.

Phenylazomalonamamidine Hydrochloride (III)—Aniline (10.5 ml.) dissolved in 6 N hydrochloric acid (65 ml.) was diazotized below 5° by the gradual addition of a solution of sodium nitrite (8.5 gm.) in water (50 ml.). 5 minutes after the addition was complete, excess nitrous acid was decomposed with urea. The diazonium solution was then poured into a solution of the amidine (II) (15.5 gm.) in water (75 ml.) and the pH of the resultant mixture brought to about 4 by the addition of a concentrated aqueous sodium acetate solution. Yellow needles of azo compound began to separate. The reaction was allowed to proceed at room temperature for several hours, then left overnight in the ice box to complete crystallization. The filtered product was washed with a small amount of water and desiccated *in vacuo*, yielding 24.2 gm., 90 per cent, m.p. 196–198°. Recrystallized from alcohol and ether, a sample was obtained which melted at 199–200°.

$C_9H_{12}ON_6Cl$. Calculated, Cl⁻ 14.67, N 28.98; found, Cl⁻ 14.49, N 28.74

Formamidomalonamamidine Hydrochloride (IV)—Phenylazomalonamamidine hydrochloride (25 gm.) was added in portions during 1 hour to a suspension of zinc dust (50 gm.) in 98 per cent formic acid (275 ml.). The mixture was agitated occasionally, but no attempt was made to dissipate the heat of reaction. After the addition was complete, the suspension was warmed on a hot plate until the residual azo color was discharged, then filtered. The filtered material was washed with formic acid and the combined filtrates concentrated to a syrup *in vacuo*. The concentration was repeated with several additions of water until the odor of formic acid was no longer evident, the temperature of the material being kept below 50° during the process. The residue was taken up in water (100 ml.) and freed of zinc by means of hydrogen sulfide. The filtrate from the zinc sulfide was now taken to a syrup *in vacuo* with repeated additions of absolute alcohol to remove water. To separate the product from the formanilide formed in the reaction, the residue was dissolved in methanol and treated dropwise with methanolic HCl until a strongly acidic reaction was shown on addition of a drop to moistened indicator paper. The addition, without delay, of a large volume of anhydrous ether precipitated the product as a gum from which the ethereal supernatant containing formanilide was shortly decanted. A few drops of water added to the product induced crystallization. The mass was broken up with 95 per cent ethanol added gradually, followed by anhydrous ether to complete crystallization. The yield was 12.3 gm. or 63 per cent calculated as the hemihydrate; m.p. 93–95°. While the product could be recrystallized in well formed crystals from aqueous ethanol

by the addition of ether, the melting points after successive crystallizations varied in either direction, due to the instability of the material in this treatment. The product obtained on the first crystallization was generally used in the imidazole cyclization without further attempt at purification. For analysis, a recrystallized sample, m.p. 95–96°, was dried over P_2O_5 at 56°.

$C_4H_9O_2N_4Cl \cdot \frac{1}{2}H_2O$. Calculated, Cl^- 18.70, N 29.56; found, Cl^- 18.37, N 29.51

Formamidomalonamamidine did not couple with diazotized sulfanilic acid, but a strongly alkaline solution warmed in a water bath for a few minutes did give an orange color in the coupling reaction. Such alkali-treated material also could be diazotized and coupled, as in the Bratton-Marshall procedure. Some formation of amino imidazole apparently takes place in alkaline solution. However, a preparative conversion of the form-amido compound to the imidazole cannot be achieved under these conditions due to extensive hydrolysis of the amidine group with the formation of formamidomalonamide (VI) as shown by the following experiment.

Formamidomalonamamidine hydrochloride (0.425 gm.) was converted to the free base by addition of 0.1 N NaOH (25 ml.) and the solution was refluxed for 1½ hours, whereupon ammonia was evolved. The solution was taken to dryness and the residue recrystallized from water. The crystals obtained, m.p. 203–205°, did not depress the melting point of formamidomalonamide (6). A yield of 52 per cent was obtained.

Aminomalonamamidine Dihydrochloride (VII)—The formamido compound (3.2 gm.) was refluxed with methanolic HCl (75 ml.) plus sufficient water added dropwise to the boiling suspension to bring about solution. After 10 minutes, the solution was taken to dryness *in vacuo*. The residue was thinned with a few drops of water and crystallized by the slow addition of absolute alcohol and ether. The product was obtained as colorless crystals, 2.5 gm., m.p. 208–209°, in a yield of 78 per cent. A sample recrystallized by the above method melted at 209–210°.

$C_3H_{10}ON_4Cl_2$. Calculated, Cl^- 37.55, N 29.62; found, Cl^- 37.22, N 29.66

4-Amino-5-imidazolecarboxamide Hydrochloride (V)—Formamidomalon-amamidine hydrochloride (2.5 gm.) was heated in a flask placed in an oil bath whose temperature was held at about 170°. The amidine melted and, within 10 minutes, most of the mass crystallized as the aminoimidazole-carboxamide. The flask was cooled and the product recrystallized from a concentrated aqueous solution by the addition of ethanol followed by ether, yielding 1.9 gm., 89 per cent, melting with decomposition at 255–256°. The melting point was unchanged by further recrystallization.

$C_4H_7ON_4Cl$. Calculated. Cl^- 21.81, C 29.54, H 3.72
        Found.   " 21.36, " 29.39, " 4.13

When the cyclization was carried out at 130°, crystallization of the molten material did not take place until it had been heated for 20 minutes. In this run a gentle vacuum was applied intermittently to facilitate the removal of water formed in the reaction.

4-Amino-5-imidazolecarboxamide was also prepared by the method of Windaus and Langenbeck (3) with modifications suggested by Allsebrook, Gulland, and Story (7). The hydrochloride obtained melted at 255–256° with decomposition and this melting point was not depressed by admixture with the hydrochloride obtained as described above. In addition, the corresponding free bases melted at 168–169° and yielded picrates melting at 237–238°; in each case mixed melting points gave no depression.

SUMMARY

A new method has been described for the synthesis of 4-amino-5-imidazolecarboxamide, an amine accumulated by *Escherichia coli* during sulfonamide bacteriostasis. This method, which is superior in ease of operation and yield to the available method, starts with ethyl cyanoacetate which is converted in five steps to the desired base in an over-all yield of 30 per cent.

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FORMATION OF 4-AMINO-5-CARBOXAMIDOIMIDAZOLE DURING GROWTH OF ESCHERICHIA COLI IN THE PRESENCE OF 4-AMINOPTEROYLGLUTAMIC ACID

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When *Escherichia coli* is grown in the presence of amounts of 4-aminopteroyl-glutamic acid just sufficient to inhibit multiplication slightly, 4-amino-5-carboxamidoimidazole accumulates in the medium, and has been isolated from it. This is the same substance which was found by Stetten and Fox[1] when this and other bacteria were grown in the presence of sulfadiazine or sulfapyridine. It was identified by Shive, *et al.*,[2] and recognized as the probable precursor in the biosynthesis of hypoxanthine.

The accumulation of the imidazole through the intervention of the anti-metabolite of folic acid is of importance in consideration of the mode of action of sulfonamide drugs and of folic acid. Thus, inhibition analysis has led to the conclusion that *p*-aminobenzoic acid participates in several reactions, of which the first to be affected by sulfanilamide derivatives is the formation of methionine, the next is concerned with purine formation,[3] and less sensitive processes, presumably the synthesis of folic acid,[4] are then retarded. On the other hand, Woods[5] has concluded that the primary action of the sulfon-amides is the inhibition of folic acid formation, and that synthesis of purines and of methionine are secondary events in which that vitamin participates. The present finding would favor the latter view. Since the folic acid antagonist leads to the accumulation of the same imidazole as do the *p*-aminobenzoic acid antimetabolites, the latter presumably act by creating a deficiency of folic acid, which in turn is responsible for the failure in purine formation.

The demonstration was conducted as follows: *E. coli* was grown in the manner of Stetten and Fox[1] except that sulfadiazine was omitted and 0.2 mg. per cc. of 4-aminopteroylglutamic acid[6] was added. Judged colorimetrically, about the same amount of diazotizable amine accumulated as when sulfadiazine

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(6) Kindly made available by Dr. T. H. Jukes of Lederle Laboratories.

was the inhibitor. Isolation of the base was accomplished according to the method described by Stetten and Fox,[1] except that 2.5 times as much mercury salt was used and the ether extraction was omitted. Final separation was made on paper strips with butanol-diethylene glycolwater solvent, in an atmosphere containing ammonia,[7] in which the imidazole showed R_F of 0.5. Identity of the isolated substance with synthetic 4-amino-5-carboxamidoimidazole[8] was established by comparison of (a) the R_F in the solvent just mentioned, (b) the absorption spectra in the ultraviolet region at pH 2.0 and 11.0, and (c) the melting points (with decomposition) of the picrate. In every case the behavior of the known and the unknown was the same.

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POST-TETANIC POTENTIATION OF RESPONSE IN MONOSYNAPTIC REFLEX PATHWAYS OF THE SPINAL CORD*

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Some processes in nervous tissue are essentially discontinuous in nature, others, like heat and carbon dioxide production, and positive after-potential, are cumulative; they tend to develop in some relation to the number of impulses carried, not infrequently to appear in measurable form only after a number of actions have been compressed into a limited time. In such conditions of activity not only are cumulative processes demonstrable in nerve, but indications of their influence may be found in the altered responsiveness of simple synaptic relays and of neuromuscular junctions. The usual sequel to a period of tetanic stimulation in junctional tissues is a more or less prolonged increase in the transmitted response to standard, iterative, but infrequently elicited pre-junctional nerve volleys into which train of volleys the tetanus has been interpolated (2, 7, 10, 11, 23, 35, etc.). The observed phenomena have been called post-tetanic facilitation, or post-tetanic potentiation; the latter designation is to be preferred.

A number of mechanisms with varying degree of experimental justification have been proposed to account for the phenomena that have been described. However it is not strictly a matter for rivalry between hypothetical alternative mechanisms (cf. in particular reference 10), for the descriptions of post-tetanic increases in response or responsiveness of various tissues preclude the possibility of fitting the phenomena to a common mold.

Post-tetanic increments of response have been encountered too in the study of spinal reflex mechanisms (5, 14, 36, 38). However, it is widely recognized that analysis of mechanism in the central nervous system at once is confronted with a new factor, the activity of internuncial chains. The potentialities for explaining prolonged effects in the central nervous system with the aid of a flexible internuncial system are enormous. On the other hand, it is logical that cumulative processes, as elementary properties of nerve tissue, should influence action there as elsewhere. Again, the aim is not to support a rivalry of hypothesis, but rather to extricate the cumulative process from supposed domination by the internuncial system, so to assess the respective rôles of discontinuous (i.e. internuncial barrage) and cumulative processes in the mechanisms for enduring alteration of transmission in the central nervous system. The description of experiments that follows relates an attempt to account for the striking post-tetanic potenti-
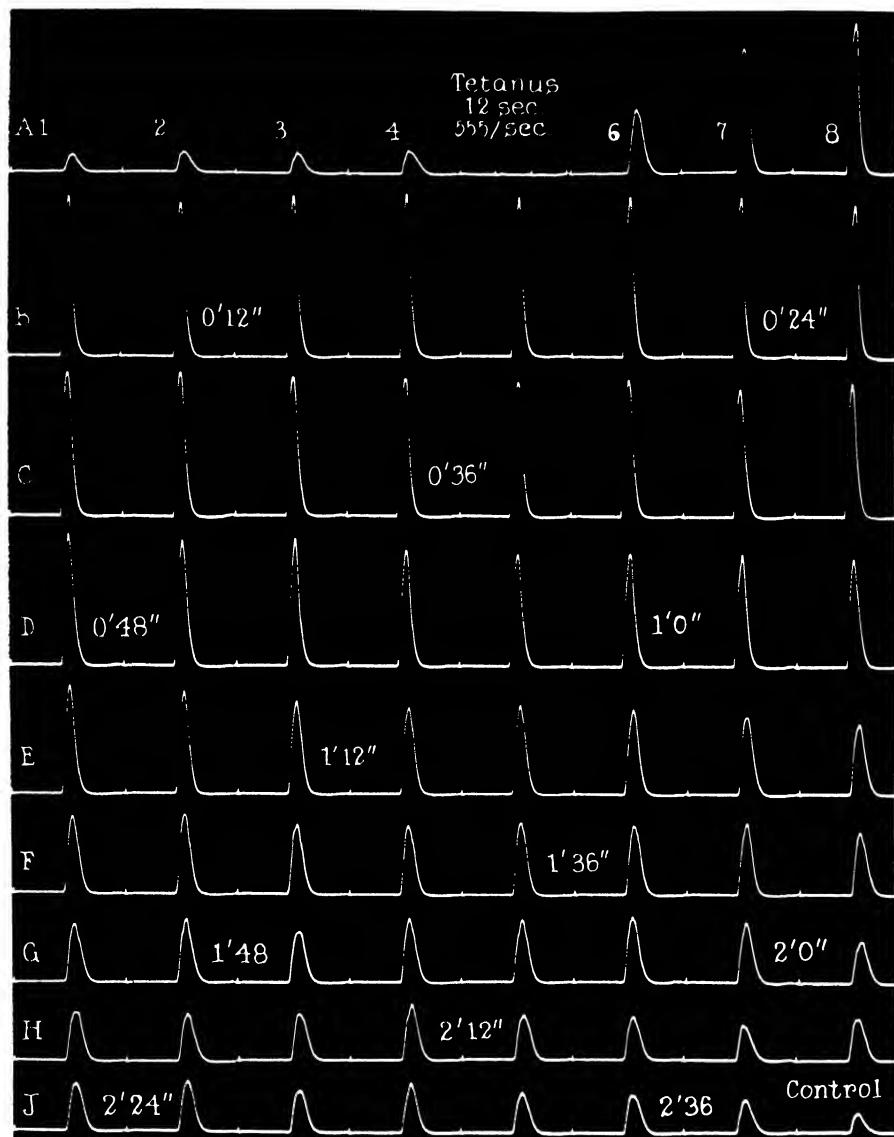
FIG. 1. Monosynaptic reflexes of gastrocnemius muscle recorded at regular intervals before and following an intercurrent tetanus to illustrate the phenomenon of post-tetanic potentiation.

ation of response that has been encountered in the monosynaptic reflex pathways of the cat spinal cord. It will be evident how closely a number of the present experiments, experimental results, and conclusions resemble those of Larrabee

and Bronk (23), whose observations concerned the activity of sympathetic ganglia.

Potentiation of Monosynaptic Reflex Transmission in the Spinal Cord.—In the experiment illustrated by Fig. 1 single shock stimuli were delivered in regular succession once each 2.4 seconds to the nerve of gastrocnemius muscle, the monosynaptic reflex discharges evoked by those stimuli being recorded from the first sacral ventral root severed distally and placed upon appropriate leads. Such reflex responses, of fairly uniform magnitude in the absence of other stimulation, are seen in A 1, 2, 3, 4, and J 8 of Fig. 1. Between the recording of responses
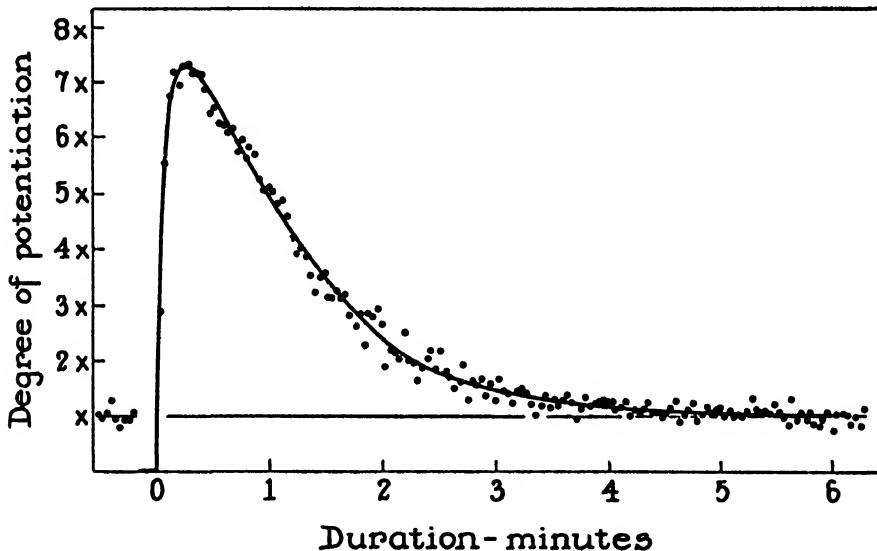
FIG. 2. Time course of post-tetanic potentiation following tetanic stimulation. Amplitude of monosynaptic reflex response expressed in multiples of the average pretetanic value is plotted against time in minutes after the end of tetanic stimulation.

A 4 and A 6 the gastrocnemius nerve was tetanized for 12 seconds at a stimulation frequency of 555 per second, following which the monosynaptic responses increased progressively (A 6, 7, 8) to a maximum (row B), thereafter slowly waning in magnitude to be still about twice the normal after more than 2 minutes (row J). By plotting amplitude of each response on a suitable time scale, a "curve of potentiation" such as that illustrated in Fig. 2 is obtained. The phenomenon of post-tetanic potentiation in monosynaptic reflex pathways is quite general, all of a number of pathways pertaining to various muscles, flexor and extensor, having behaved in similar fashion in similar circumstances of stimulation.

A cumulative process underlying post-tetanic potentiation of response might reside in the presynaptic elements or the postsynaptic elements of the monosynaptic reflex arc, or it might be assumed that extracellular agents, excitor

substances or appropriate ionic species, liberated into the synaptic environs to persist there for due time, by their action could augment transmission. Finally, as earlier stated, when junctions within the central nervous system come under
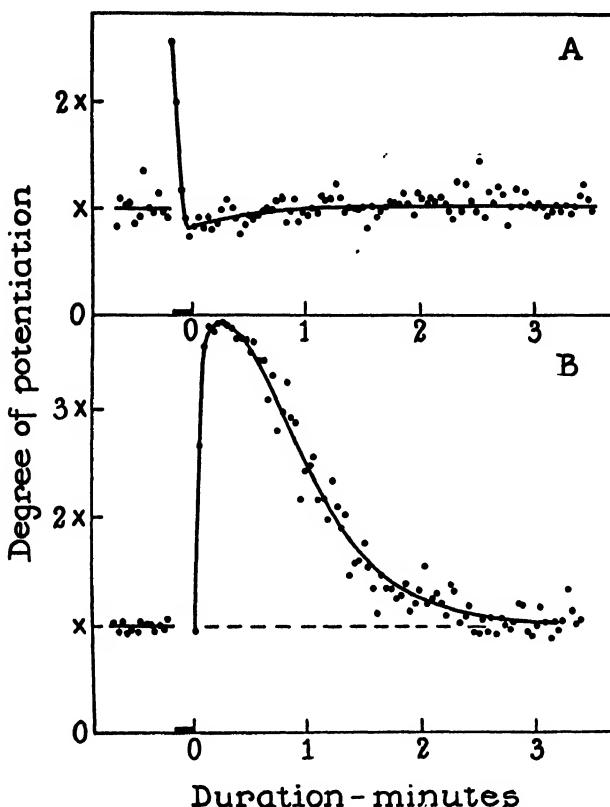
Fig. 3. Absence of post-tetanic potentiation when tetanus and test stimulations are applied to different nerves. A, Monosynaptic reflexes of knee flexor muscles before, during, and after tetanization of the sural nerve. Typical flexor reflex facilitation of the test response occurred during the tetanus, to be followed by a period of depression. In contrast, B shows the typical potentiation following tetanization of the knee flexor muscle nerves employed for test stimulation.

observation, even though they constitute a monosynaptic pathway, the possible play, through convergent chains, of internuncial barrage, must be considered.

The need for experimental elimination of internuncial barrage as a factor for post-tetanic potentiation in monosynaptic reflex paths stems from the fact that tetani induce threshold changes in the stimulated fibers, the effects of which must be obviated by the use of shocks supramaximal for the group I afferent fibers of the monosynaptic reflex pathway. As a consequence of this necessity,

higher threshold afferent fibers, possessing extensive ramification to the internuncial pools, unavoidably are stimulated. Interneurons thrown into activity by stimulation of muscle nerves in general are excitatory to flexors and inhibitory to extensors. That post-tetanic potentiation of flexor (Fig. 3 B) and extensor (Fig. 2) monosynaptic reflexes is similar, therefore, provides the initial clue to the effect that internuncial activity is not responsible for potentiation.

Unfortunately it is neither possible to tetanize monosynaptic paths in proven isolation, nor to tetanize the internuncially relayed pathways of a given muscle nerve leaving its monosynaptic paths at rest. Hence the obvious and most direct controls for the participation of interneurons cannot be applied. However one can tetanize a variety of afferent nerves known to relay through internuncial
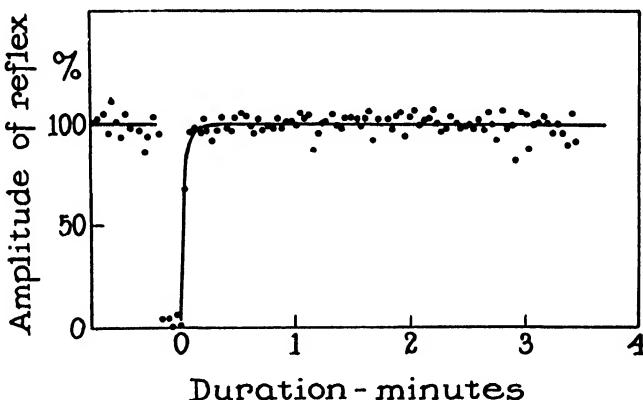
FIG. 4. The monosynaptic reflex response of an extensor muscle was inhibited during tetanic stimulation of the ipsilateral peroneal nerve. At the close of the tetanic period the monosynaptic reflexes returned to normal amplitude without displaying potentiation.

pathways to the motoneurons that form part of any given monosynaptic test system. For instance, Fig. 3 A plots the amplitude of regularly evoked monosynaptic reflex responses of semitendinosus motoneurons before, during, and following tetanic stimulation of the sural nerve. As would be expected the monosynaptic responses were facilitated during the tetanus, but the only effect in the post-tetanic period was a slight depression that passed off during the first minute. Figure 3 B, practically a continuation of Fig. 3 A, shows the potentiation of semitendinosus monosynaptic reflex responses that followed tetanization of the semitendinosus nerve itself. Another experiment for which an extensor monosynaptic system was tested is shown in Fig. 4. Monosynaptic reflex responses to stimulation of the gastrocnemius nerve were elicited at regularly recurring intervals, the interpolated tetanic stimulation being applied to the peroneal nerve. During that tetanus the gastrocnemius test reflex was in-

hibited and, at the close of the tetanus, it required several seconds for the gastrocnemius reflexes to regain normal amplitude, but once this was accomplished no further change ensued.

The foregoing experiments demonstrate that tetanic and monosynaptic test stimulations must be applied to the same nerve for the characteristic post-tetanic potentiation of reflex discharge to ensue. This fact makes participation of internuncial activity an unlikely cause of post-tetanic potentiation of monosynaptic reflex discharge. However it is not disproved uniquely thereby.

Post-Tetanic Responses through Plurisynaptic and Monosynaptic Pathways Contrasted.—Fig. 5 presents the result of an experiment in which reflex discharge through internuncially relayed pathways was observed before, during, and subsequent to a tetanic stimulation of those same pathways. The sural nerve was afferent for all stimulations, the reflex discharges being recorded from the first sacral ventral root. Because of variability in the response to single shocks a number of control observations (A 1 to C 2 in Fig. 5) preceded the tetanus. Between the recording of C 2 and C 4 the sural nerve was tetanized for 12 seconds at a frequency of 575 per second. From the records that follow it is seen that the effect of tetanization on subsequent transmission was slight and evanescent. Thus, while it is clear from Bernhard's experiments (1) that interneuron chains act to produce the characteristically dispersed flexor reflex discharges of single shock excitations, there is no evidence for prolonged self-perpetuating activity of the sort that would have to be postulated to account for post-tetanic potentiation. This is not to deny that interneurons may act for the perpetuation of states of activity in other circumstances, it merely indicates that the conditions for post-tetanic potentiation are not of necessity those in which neuron chains can maintain a barrage of the motoneurons.

It is of some interest to inquire into the failure of plurisynaptic chains to reveal in a convincing manner evidences of the potentiating action. In explanation of the failure one might suppose the underlying action not to occur, or that having occurred some part of the system cannot respond in a revealing manner. The former supposition is tantamount to postulating drastically different fundamental properties for the nerve elements entering into monosynaptic and plurisynaptic reflex systems. Such a postulate seems unlikely when one recalls that sympathetic ganglia exhibit post-tetanic potentiation (7, 23) remarkably like that in spinal monosynaptic systems despite the fact that fibers of quite different properties are concerned in transmission through ganglia and spinal cord. The latter supposition has more merit. It is a logical necessity for potentiation to occur that a subliminal fringe exist in the test system at the junctions where the post-tetanically reinforced action is exerted. Since tests by the use of flexor monosynaptic reflexes reveal at the final common path an adequate subliminal fringe "surrounding" the discharge zone when the tested action is provoked by a sural nerve volley (26) or even a peroneal nerve volley (1), it follows that the defect underlying the failure of plurisynaptic systems clearly to exhibit post-tetanic
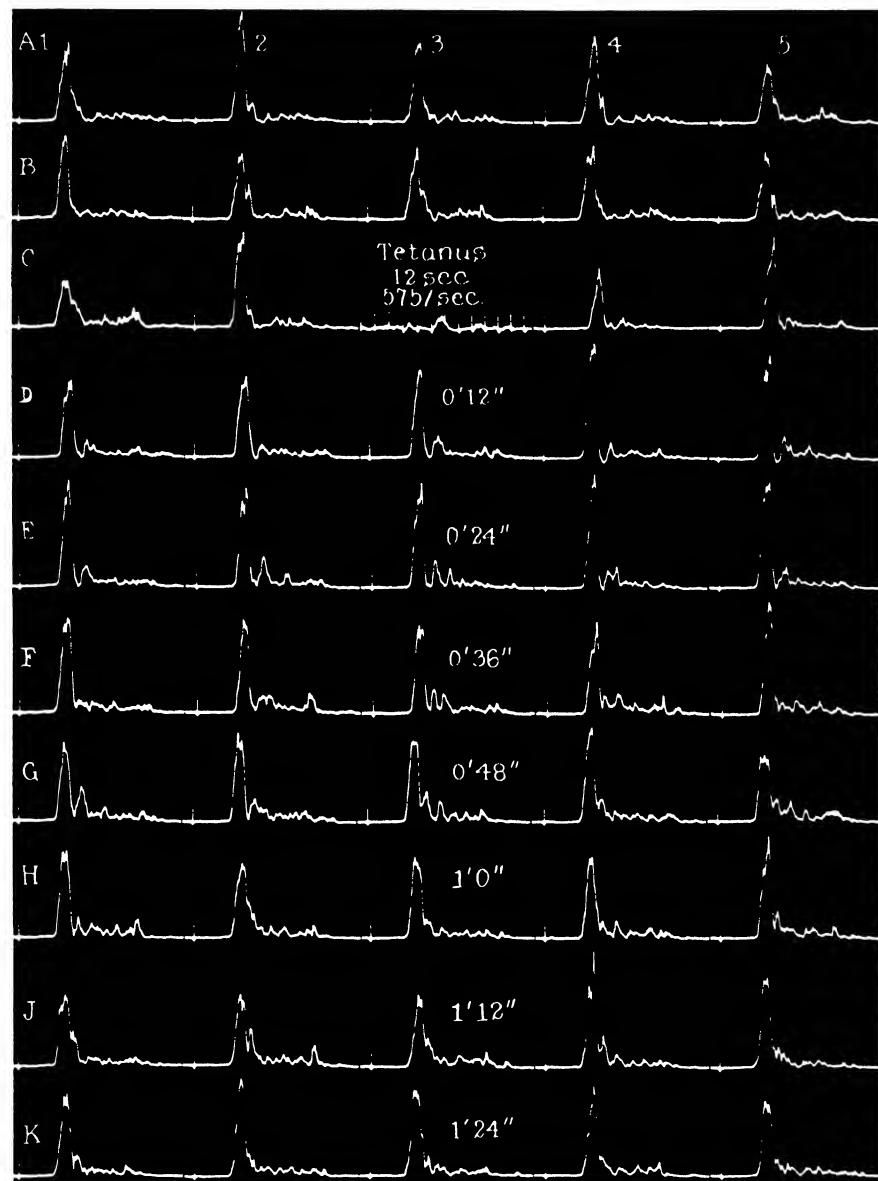
FIG. 5. An example of the relatively insignificant change in plurisynaptic reflex discharges following an intercurrent tetanization of the afferent nerve (sural).

potentiation lies not at the synaptic junctions with motoneurons, but upstream. It is of interest in this connection that Huges and. Gasser (21) could find no

facilitation of negative intermediary potential in the presence of marked facilitation of the flexor reflex. It is not unlikely that the failure of negative intermediary potentials to exhibit facilitation, and the failure of the plurisynaptic reflex paths to display undoubted post-tetanic potentiation, share a common basis among the properties of the initial internuncial relays.

Post-Tetanic Effects Tested by Antidromic Volleys.—A number of possible mechanisms for post-tetanic potentiation may be put to test by means of direct observation of the responses of motoneuron somata. In a certain number of motoneurons antidromic impulses fail to penetrate the soma, with the result that the somatic action potential evoked by a maximal antidromic volley, in an otherwise "resting" spinal cord, is less than full size. The block to antidromic conduction is labile and easily relieved by maneuvers that facilitate even mildly the monosynaptic reflex response of the motoneurons. For instance the relatively mild internuncial bombardment of lumbosacral motoneurons during the course of a long spinal reflex (24, 30) is effective in relieving block (25), and it can be shown in this system that the degree of relief is proportional to the intensity of the internuncial barrage as measured by facilitation of a monosynaptic reflex test (29). Brooks and Eccles (9) have shown the relief of block by group I afferent volleys closely to resemble in time course the residual facilitation of motoneurons by similar volleys (27). In short, the degree of penetration into motoneurons of antidromic volleys is a delicate test for the working of excitatory influences upon those motoneurons.

Several hypothetical post-tetanic excitatory events should be uncovered by the use of antidromic test volleys. Among these would be any enduring intrinsic rise in excitability of the motoneurons themselves, a possibility raised by the observations of Kleyntjens on frog spinal cord (22). Such an effect might be the result of the motoneurons having fired a number of impulses as seems to be the case in Kleyntjens' experiments (although the usual sequel to discharge in the cat is depression) or the result of some change, not associated with firing but developed under the influence of repetitive impingement of presynaptic impulses. Another hypothetical event that should influence response to antidromic volley tests would be the release by presynaptic action into the environment of the motoneuron somata of an extracellular agent that might modify for several minutes the excitability of the motoneurons. Additionally the antidromic volley test forms another means of controlling the possibility of action by self-perpetuating internuncial activity.

Admittedly if the somatic responses to antidromic volleys were to be augmented in the period following a presynaptic tetanus, it would not be possible without other evidence to differentiate between the mechanisms mentioned. However, absence of a post-tetanic augmentation would speak against them all and weigh heavily in favor of the remaining possibility: that an enduring change in the properties of the presynaptic fibers following intense activity alters some essential character of subsequent impulses conducted by them.

Fig. 6 illustrates an experiment performed in examination by means of anti-dromic volleys of the after-effects of a presynaptic tetanus. A segmental reflex pathway was employed, the test stimulations being delivered to a ventral root whereas the tetanic stimulation, of 12 seconds duration at a frequency of 575 per second, was directed to the ipsilateral dorsal root of the same segment. Re-
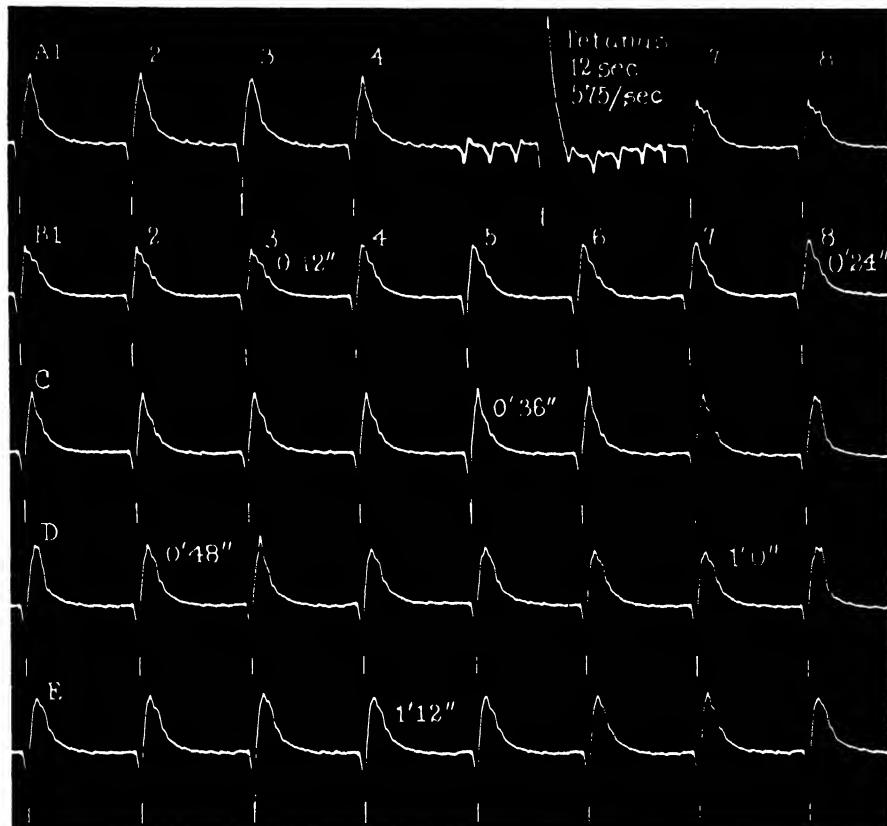
FIG. 6. Increase, during a presynaptic tetanus, of the soma response to antidromic volleys followed by depression during the early post-tetanic period.

cordings were obtained through the use of a microelectrode inserted to the ventral horn. In parallel experiment the segmental monsynaptic reflexes elicited by stim-uli to the tetanized dorsal root were seen to be potentiated for several minutes following comparable tetanic stimulation.

Responses A 1, 2, 3, and 4 of Fig. 6 anteceded the period of tetanic stimula-tion, and serve to establish the course of somatic responses elicited at regular intervals of 2.4 seconds. Between responses A 4 and A 7 is a record, obtained during the presynaptic tetanus, in which may be seen the regularly recurring

action potentials of the presynaptic impulses, and a single interpolated anti-dromically evoked somatic response of the motoneurons. This latter reveals the characteristic increment in response that resulted from the impingement of pre-synaptic impulses. Following the tetanus (A 7, 8 *et seq.*) the somatic responses were depressed rather than enhanced. These results not only lend support ex-clusively to the hypothesis of change in the presynaptic fibers, they further show that the potentiation of monosynaptic reflex discharge develops in the face of a post-tetanic depression in the postsynaptic elements (*cf.* also reference 23). Depression of the sort illustrated by Fig. 6 undoubtedly resembles that seen in Fig. 3 A, and, as will be seen later, accounts in 'part for the character of the rising phase of potentiation.

Potentiation of Facilitation and Inhibition.—Impulses that arise in the group I afferent fibers of a given muscle not only initiate monosynaptic reflex discharge of the motoneurons supplying that muscle, but, by direct impingement, they facilitate action in the monosynaptic paths of synergist muscles, and inhibit action in the monosynaptic paths of antagonist muscles (28). If, as the preceding evidence suggests, the mechanism underlying potentiation may be traced to some altered property of the presynaptic afferent fibers then the facilitator and inhibitor actions of those fibers presumably should be potentiated in the post-tetanic period along with their transmitter action. Tests of this expectation have been fashioned according to the following argument. Changes in the magnitude of a reflex discharge reveal themselves directly to the recording system applied to the motor axons, not so changes in facilitation and inhibition for the examina-tion of which a standard test reflex is required. Furthermore, it is now clear that the only reflex actions potentiated following an afferent tetanus are those in-stigated by subsequent stimulation of the tetanized afferent fibers. Thus it follows on two counts that demonstration of a potentiation of facilitation or inhibition requires the establishment of two systems of test shocks, the first of these, ap-plied to the tetanized nerve, to explore the after-effects of the tetanus, the other, applied to the nerve of a synergist or antagonist muscle, to reveal, by changes in the monosynaptic reflexes of those muscles, the degree of facilitation or in-hibition derived from test stimulation of the tetanized nerve. Since the intensity of facilitation or inhibition of a reflex in a two-shock system (the response to the second testing the action of the first) is a function of the time interval be-tween the shocks, then for the combined shocks to be a valid test for the influence of prior tetanization it is obvious that the individual shocks of the pair, through-out any given experiment, must bear a constant temporal relation to each other.

In the experiment illustrated by Fig. 7 the nerves of the two heads of gastroc-nemius muscle were arranged for independent afferent stimulation. One nerve was stimulated by single shocks, once each 2.4 seconds, to evoke a regular suc-cession of monosynaptic reflex discharges. For alternate applications shocks to that nerve were delivered in isolation, magnitude of the monosynaptic responses

so elicited being represented in Fig. 7 by dots, and in combination with an ante-
cedent shock to the other gastrocnemius nerve, the magnitude of the facilitated
monosynaptic responses that resulted being represented in Fig. 7 by circles.
Thus, when the one shock was given, the size of the resulting monosynaptic
reflexes tested for direct effect imposed by tetanization of the other nerve,
whereas when the two shocks were given in sequence the size of the facilitated
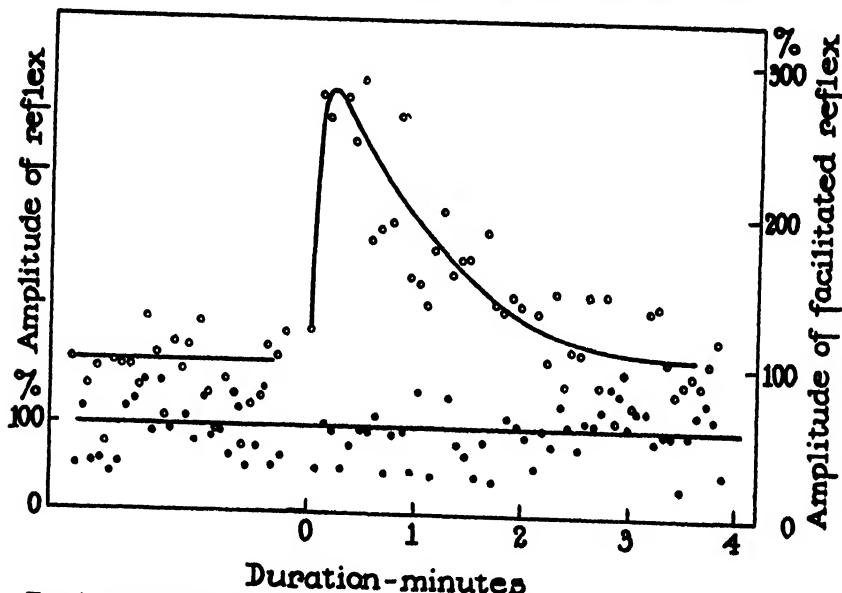
FIG. 7. Following tetanization of the nerve to one head of a muscle (gastroc-
nemius) the facilitating action of a volley in that nerve upon the monosynaptic reflex
responses of the other head is potentiated in characteristic time course (circles).
However there is no direct effect of tetanizing the nerve to one head upon the mono-
synaptic reflex response of the other (dots).

monosynaptic reflexes tested the after-effect of tetanization upon the facilitatory
action of subsequent volleys stimulated in the recently tetanized nerve.

The observations recorded in Fig. 7 show that tetanization of the nerve to one
head of gastrocnemius is without direct potentiating effect upon transmission
of monosynaptic reflexes arising in the nerve to the other head of gastrocnemius.
But the facilitating action that a volley in the nerve to one head exerts upon
transmission through the monosynaptic pathway of the other head, subsequent
to tetanic activation of the "facilitator" nerve, is potentiated in a manner and
degree that reproduces the course of post-tetanic potentiation of monosynaptic
reflex discharges. Comparable result has been obtained in experiment with the
pathways of semitendinosus and biceps femoris posterior.

Potentiation of inhibitory action has been studied in the same manner as potentiation of facilitation, the only and necessary difference in procedure being the selection for stimulation of nerves to an antagonist rather than a synergist muscle pair within the myotatic unit. Illustrated by Fig. 8 is an experiment that utilized the nerve supply to the pretibial muscles, tibialis anterior and extensor longus digitorum, as afferent for the measured monosynaptic test reflexes, and
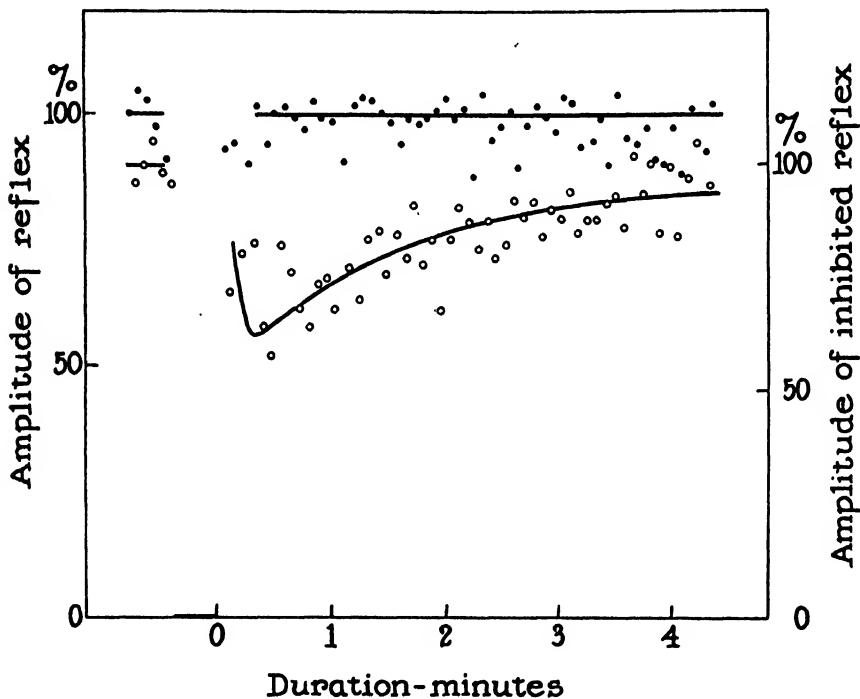
FIG. 8. A tetanus of the nerve to one muscle (gastrocnemius) potentiates the inhibitory action of subsequent volleys in that nerve upon the monosynaptic reflex responses of antagonists (ankle flexors) within the myotatic unit (circles). Tetanization of the one nerve again is without direct effect upon the monosynaptic reflex evoked by stimulation of the other nerve (dots).

the nerve to gastrocnemius as a source of inhibitory volleys. With the two shocks in close temporal approximation the intensity of inhibition was not great. Once the amplitude of control (dots) and inhibited (circles) monosynaptic responses was established, the gastrocnemius nerve was tetanized, following which the inhibitory action of subsequent gastrocnemius nerve volleys was potentiated for several minutes, although transmission through the monosynaptic pathways of the pretibial muscles was not influenced directly.

The experiments of Figs. 7 and 8 not only support the notion that the tetanic

aftermath is localized to the afferent fibers tetanized, they demonstrate also that any of the tangible synaptic actions, regardless of direction, of impulses in those afferent fibers are potentiated following a tetanus. This being so, the simplest conclusion would be that the change underlying post-tetanic potentiation influences the afferent fiber generally, rather than merely the terminals (23), probably in such a way that the impulses engendered in it subsequent to tetanization are greater than normal. Apparently Boyd (2), studying post-tetanic decurarization of the cat's tongue, was the first to suggest the possibility that
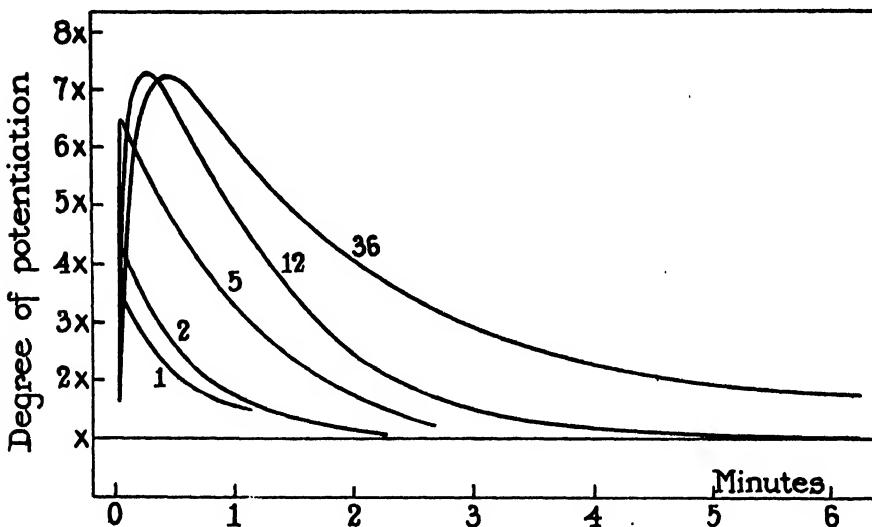
FIG. 9. Time course of post-tetanic potentiation after various durations of repetitive stimulation (1, 2, 5, 12, and 36 seconds) at a constant frequency (*ca.* 500 per second).

post-tetanic change in the nerve impulses was responsible. His suggestion, often dismissed, according to the present indications should be reinstated.

Influence of Changed Duration and Frequency of Stimulation upon the Course of Post-Tetanic Potentiation.—Considerable information as to the properties of a cumulative process such as underlies post-tetanic potentiation may be obtained by varying either the duration or frequency of stimulation. Fig. 9 plots the course of potentiation following tetani of varying durations, but of constant frequency. With the shorter tetani (1 or 2 seconds) the first post-tetanic response revealed the maximum enhancement. Following the 5 second tetanus maximum was attained on the second and third post-tetanic responses. As the tetanus was further lengthened (12 and 36 seconds) the rising phase of potentiation was progressively slowed. The earlier experiments (Figs. 3 A and 5) suggest, in explanation, that post-tetanic potentiation develops in the face of an opposing depression

that in turn bears its own peculiar relation to the duration of stimulation. If that opposing depression is due to the summation of subnormality in postsynaptic structures, then of course the ability of those structures to follow the tetanus frequency becomes an added factor modifying the relation between depression and severity of tetanization. By the nature of the experiments of Figs. 3 and 9 it is not possible to locate the depressed structures, but in the experiment of Fig. 5, in which antidromic volleys were employed to test the effects of a presynaptic tetanus, the early post-tetanic depression of somatic responses proved the motoneurons at least to be in a' depressed state. Comparable depressions and slowing of the rising phase of post-tetanic potentiation in sympathetic ganglia have been described by Larrabee and Bronk (23).
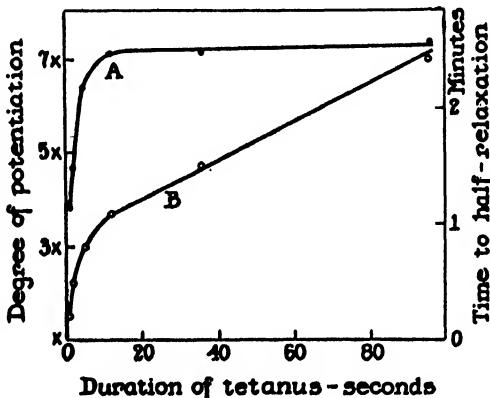
FIG. 10. Intensity (A) and duration (B) of post-tetanic potentiation of a monosynaptic reflex as a function of duration, in seconds, of a tetanus at constant frequency.

Another feature of post-tetanic potentiation is the fact that a ceiling is reached with tetanic stimuli of about 10 seconds duration or greater. Once the ceiling is reached, further prolongation of a tetanus results in more prolonged potentiation. This effect can be seen in the plots of Fig. 9 and to better advantage possibly in Fig. 10. Curve A of Fig. 10 expresses as a function of tetanus duration the degree of potentiation of the largest single response recorded during the post-tetanic period. Curve B similarly relates duration of potentiation, measured by the time to half-relaxation, to the duration of tetanic stimulation. It is seen that the magnitude and duration of post-tetanic potentiation were increased in parallel fashion with lengthening of the tetanus until the ceiling was reached. Thereafter, continuing increase in the duration of potentiation fell into an approximately linear relation with the duration of the applied tetanus.

Figs. 11 and 12 present the results of two experiments in which was investi-
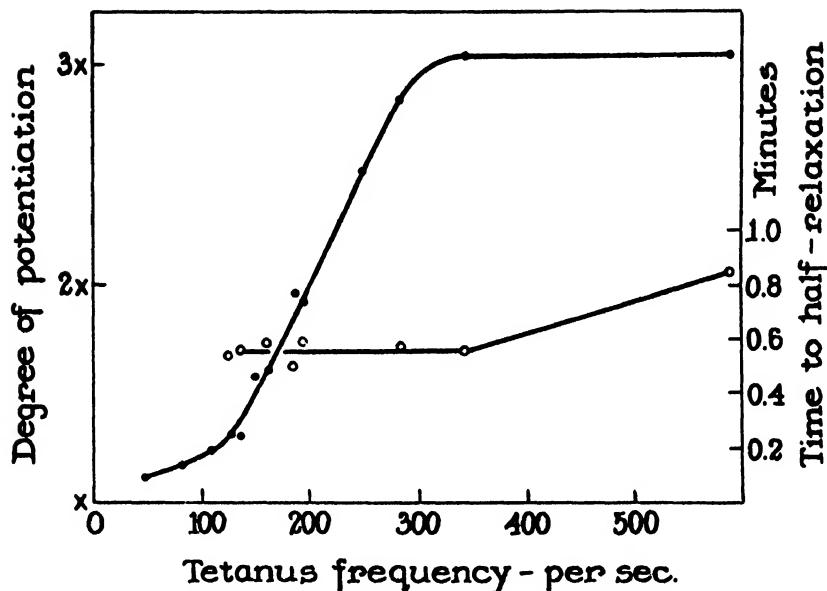
FIG. 11. Intensity (dots) and duration (circles) of post-tetanic potentiation of a monosynaptic reflex as a function of tetanus frequency, duration of stimulation being held constant at 12 seconds.
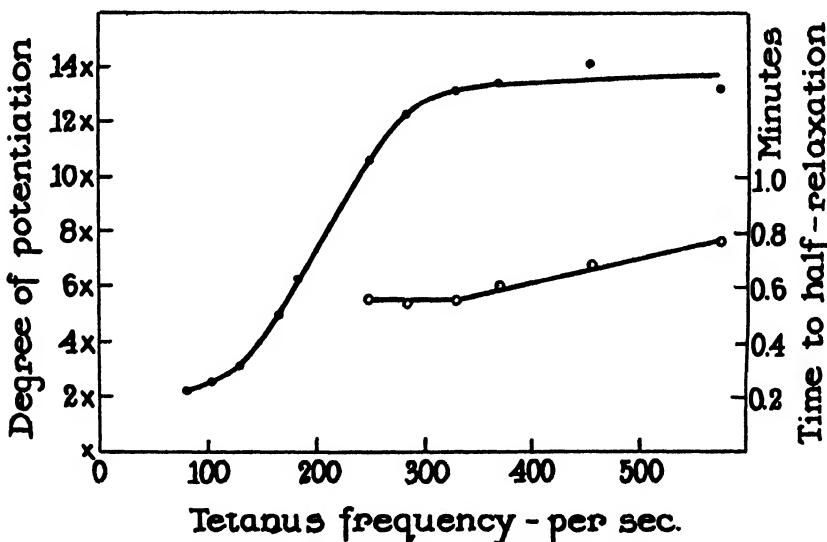
FIG. 12. As in Fig. 11, but from another experiment, a comparison of the two experiments illustrating an inverse relation between degree of potentiation and initial size of the reflex discharge.

gated the influence upon post-tetanic potentiation of change in frequency of the applied tetanus. It is of interest to compare the two experiments, for in one (Fig. 11) a fairly large monosynaptic reflex resulted from the test stimulation applied in isolation, and the maximal degree of potentiation was correspondingly low (3 ×), whereas in the other (Fig. 12) the relatively small test reflex response at the peak of potentiation was multiplied 14-fold. .

At the lower frequencies of tetanic stimulation measurement of the degree and duration of post-tetanic potentiation is difficult, for the normal variability of reflex response can obscure small effects, particularly when there is no opportunity to overcome the effect of variability by the use of repeated measurement. In one experiment definite potentiation of monosynaptic reflex response was recorded following stimulation at a frequency of 15.5 per second. There seems to be no readily available explanation for the initial upward concavity in the curves relating degree of potentiation to tetanic frequency. In one experiment the concavity was not obvious. Presumably other interfering, but unresolved, phenomena influence the post-tetanic response.

Following tetani in the frequency range between about 100 and 300 per second the degree of potentiation was found to vary linearly with the tetanic frequency, but with further increase in frequency the degree of potentiation approached a maximum. There was no significant change in duration of the post-tetanic potentiation with increasing frequency of stimulation until the ceiling in degree of potentiation was reached, but further increase in the tetanization frequency prolonged the after-effect.

The observations of Figs. 10 to 12 are of interest because they point unmistakably toward certain resemblances between the phenomenon of post-tetanic potentiation and the positive after-potential developed by tetanized nerves (15, 17, 19). Discussion of the similarities between these events is the more pertinent because of the fact that some process in and restricted to the tetanized presynaptic fibers themselves has been shown to underlie post-tetanic potentiation.

That the after-potentials of nerve may last for several minutes is well known. Gerard (19) and Gasser (15) found them to persist in frog nerve for 10 to 15 minutes. Unfortunately for the present purpose there has been little study of the prolonged positive after-potential as it appears in mammalian nerve. However, Gasser and Grundfest (17) have shown that the degree of after-positivity in mammalian nerve (as in frog nerve) increases to a ceiling with increasing severity of tetanization, after which a prolongation of the after-positivity reflects the further increase in tetanization. Furthermore, as concerns duration they gave the following figures as representative: a 10 second tetanus resulted in after-positivity that regressed to half-value in 15 to 30 seconds; after a 30 second tetanus half-relaxation required more than 1 minute. Comparison of these properties of after-positivity in mammalian nerve tissue with those re-

quired of the hypothetical process responsible for the occurrence of post-tetanic potentiation confirms the notion of close correspondence. In the circumstances, and considering that the number of unrelated coexistent and parallel events in a nerve fiber must be limited, it seems not unlikely that the cause of post-tetanic potentiation is tied to the positive after-potential process.

Observations on Presynaptic Fibers in Relation to Post-Tetanic Potentiation.— The observations and correlations that have been described lead to the following specific hypothesis as to the mechanism of post-tetanic potentiation in monosynaptic reflex systems of the spinal cord. (I) Following tetanic stimulation the stimulated afferent fibers enter a period of prolonged positive after-potential. (II) During the after-potential the intramedullary collaterals must be reasonably uniformly hyperpolarized, otherwise an external field would be established which might be expected to influence the excitability of nearby or synaptically related postsynaptic units. (III) The spike potential per fiber during the phase of post-tetanic hyperpolarization is increased over the resting, or pretetanic, value. (IV) Post-tetanically exalted afferent impulses upon reaching the afferent termini, or such other places from which they may act for transmission, and for facilitation or inhibition of transmission, do so with intensity enhanced roughly in proportion to the amount by which they are exalted. (V) The stimulation frequency requirements are such that one might conclude not unreasonably that positive after-potential could operate for increased responsiveness in the normal functioning of the nervous system.

The several steps of the foregoing hypothesis for the most part either have been tested, or are amenable to test. They may be considered seriatim.

(I and II) Most pertinent for present purposes are the observations of Woolsey and Larrabee (38) on positive after-potential in dorsal roots. Unfortunately these have never appeared *in extenso*. However, those authors observed[1] positive after-potentials lasting, in a dorsal root, for "more than a minute" following tetanic stimulation. The potentials so recorded were undoubtedly those of the extramedullary dorsal roots themselves, and not electrotonic extensions from the intramedullary projections. Recording within the spinal cord of a "P 2" positive after-potential seems impossible *a priori* for the available evidence indicates the absence of any significant external field accompanying the hyperpolarization. However, the fact that Woolsey and Larrabee (38) recorded augmented D.R.V. dorsal root potentials (31) in a recently tetanized root may be taken as evidence that the intramedullary projections of those roots were hyperpolarized in the post-tetanic period.

(III) The postulate of relative increase in the spike potential when written upon positive after-potential has developed out of an interesting history. Increase in nerve activity following a tetanus apparently was noted first by Wal-

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[1] Records of their observations were presented at the 1940 meeting, in New Orleans, of the American Physiological Society.

ler (37). Richards and Gasser (34, *cf.* also reference 18) found an increased spike potential when frog C fibers were stimulated during the after-positivity of an antecedent response. Later, increase in mammalian C fiber spikes during the after-positivity created by a tetanus was described by Grundfest and Gasser (20). Subsequently a number of investigators confirmed the effect in mammalian C fibers and in sympathetic ganglia. At the same time increase in C fiber negative after-potential intercurrent upon positive after-potential was
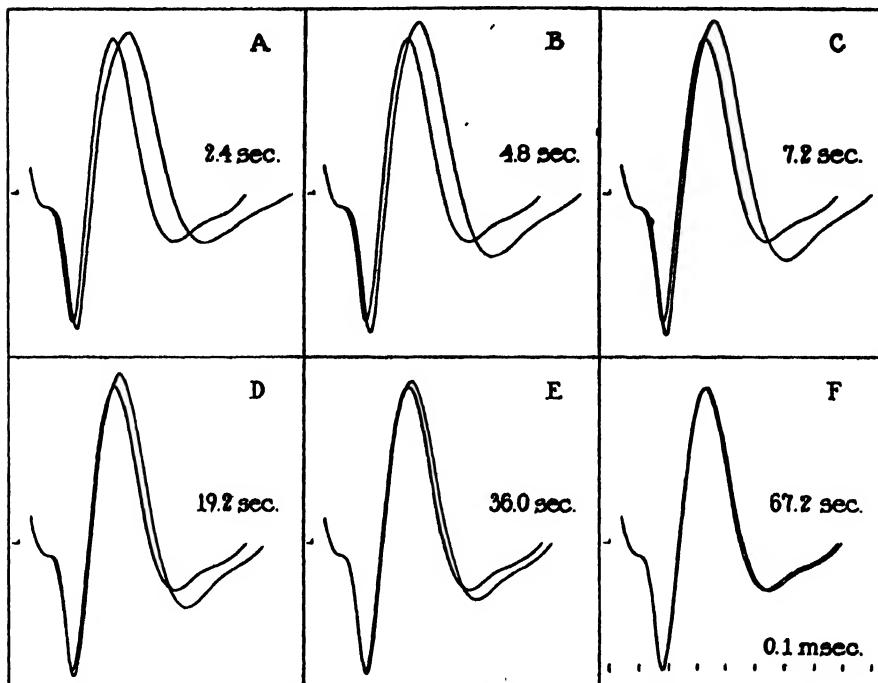
FIG. 13. Change in the recorded action potentials of afferent volleys at the stated times following the end of tetanic stimulation. Each observation, A to F, includes a control record and a post-tetanically altered record superimposed by tracing.

documented. Likewise Gasser has shown (16) an increase in mammalian A fiber negative after-potential on stimulation during the prolonged after-positivity following a tetanus. But as far as the present author is aware increase in the mammalian A fiber spike in the post-tetanic period has not been described. Obviously then, proposition III of the foregoing hypothesis must be put to test.

Fig. 13 presents the results of an experiment in which the intramedullary spike potentials of afferent volleys were recorded before and after a dorsal root tetanus. In order clearly to indicate the changes consequent to tetaniza-

tion, each observation (A to F of Fig. 13) of a post-tetanic spike is superimposed upon a spike potential recorded prior to tetanization. It is seen from the records that conduction velocity following the tetanus was subnormal in progressively diminishing degree, and that the spikes, in fulfillment of expectation, were increased over the pretetanic size. True, one cannot confirm by direct experiment the parallel existence of hyperpolarization in the intramedullary afferent fibers, and so prove the parallelism between positive after-potential and increase in spike size. Still the body of circumstantial evidence for the association is reasonably complete. Even in the unlikely event that positive after-potential ultimately should prove not to be the mechanism of increase in the spike potentials of the intramedullary afferent projections, the fact of the post-tetanically increased spikes remains to satisfy the requirement for increase of presynaptic action in support of potentiation in the reflex pathways.

It should be noted that increase in afferent spikes following a tetanus has been seen with any location of a microelectrode along the collateral projections in the spinal segment under observation, and in the extramedullary segment of a tetanized dorsal root. Potentiation of the "focal synaptic potential" of Brooks and Eccles (8) also has been noted.

(IV) This proposition requires some comment. It is important to emphasis, in accordance with the observations above, that the altered property involves the extramedullary and intramedullary course of the fibers, not merely the synaptic terminations. This being so, it follows that any action at or near fiber terminations that is tied to the nerve impulse, and this presumably includes all excitatory and inhibitory synaptic phenomena, should be enhanced. The experiments illustrated by Figs. 7 and 8 stand in support of this notion. As far as the present experiments go there has been no suggestion of reversal of action, as for instance has been seen when activity is intensified by strychnine (33) rather than prior tetanization.

From another point of view it is probable that concentration of attention upon the presynaptic terminations rather than the fibers as a whole has delayed recognition of the rôle played by exalted impulses in the causation of post-tetanic potentiation. However, it should be noted that Eccles (12) has made the pertinent suggestion recently that positive after-potential of the preganglionic fibers might provide an explanation of the phenomenon in sympathetic ganglia as described by Larrabee and Bronk (23).

Proposition IV includes the postulate of approximate parallelism between the alteration in presynaptic fibers and the potentiation of reflex response to action in those presynaptic fibers. This can be tested by comparing the temporal course of increase in spike size with that of potentiation. It is important for such a test that the two events be measured simultaneously, for small variations occur from experiment to experiment. The requirement is satisfied by use of the double-beam oscillograph with leads arranged to record simul-

taneously the afferent spike potential and the monosynaptic reflex response. Fig. 14 presents the results of an experiment performed in the manner indicated. The upper plot charts the course of reflex potentiation, the lower that of increase in afferent spike potential. It is clear that the two responses, presynaptic and postsynaptic, varied in parallel during the post-tetanic period.

Proposition V deals not with the mechanism of post-tetanic potentiation, but rather with the question of its potential significance as a means for increased
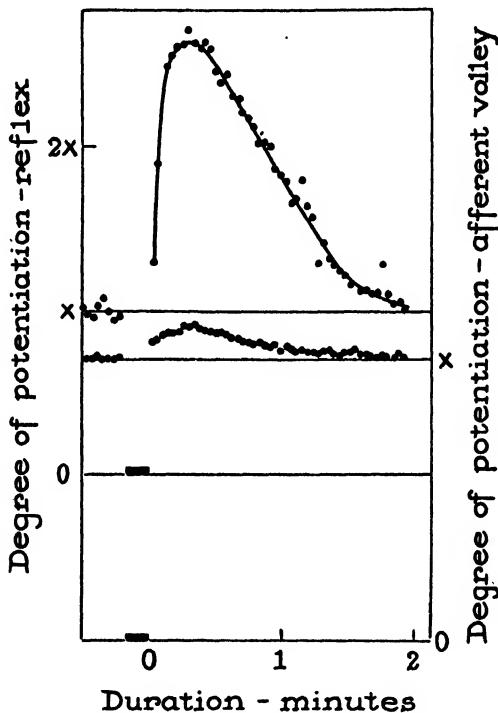
FIG. 14. Changes in amplitude of presynaptic (below) and postsynaptic (above) volleys of a monosynaptic reflex pathway simultaneously recorded, following a presynaptic tetanus.

response in the naturally activated nervous system of the intact organism. The decision between physiologically significant mechanism and laboratory curiosity depends primarily on the relation between the frequencies normally encountered and those requisite for potentiation.

Definite potentiation has been encountered following a train of 176 volleys at 15.5 per second, or, in another instance, a train of 372 volleys at 31 per second. At no time when frequencies as low as 50 per second were employed did post-tetanic potentiation, in degree sufficient to rise well above the random variation, fail to occur. Since there now appears to be little doubt that the mus-

cle spindle is the afferent end-organ to the monosynaptic reflex paths, and, since it is these same paths that exhibit the phenomenon of potentiation, the observations of Matthews on afferent discharge frequencies of end-organs in muscle (32) are the most pertinent for the purpose of comparison. In making that comparison responses from the A type of ending should be chosen, and the frequencies during static rather than phasic stretch considered. Even with this last restriction imposed the frequencies reported by Matthews as resulting from quite modest loadings are in excess of those required for potentiation. Hence it is possible that impulse potentiation by means of positive after-potential could represent a normal mechanism for increased response in the spinal cord.

Potentiation and After-Discharge.—These functions are unrelated. Whatever may be the mechanisms of after-discharge, its occurrence demands a continued action of, or upon, the postsynaptic neurons such as has been shown not to exist in the circumstances that support post-tetanic potentiation of the type herein described.

Some Prior Observations on Post-Tetanic Increase of Response in the Spinal Cord.—There exist a number of instances in which enhanced responses have been obtained following tetanic reflex stimulation. Experimental conditions have differed widely as have the results. A study of the individual manifestations of increased response leaves no doubt that the phenomenon presently under consideration does not present the only mechanism available to the spinal cord for achieving a superficially similar end-result. However self-evident this statement may seem, nevertheless consideration of a few examples is in order, particularly with respect to those studies in which test stimulation followed tetanic stimulation of the same afferent fibers.

The use of single shock test reflexes for gauging the responsiveness of the reflex arc was introduced by Sherrington and Sowton (36), who described an augmentation of such reflexes lasting on a declining scale for 16 seconds following the close of a 5 second tetanus. From the nature of the reflex test and the relative brevity of the effect, one might conclude that some factor other than increased afferent impulses determined the experimental finding. Also, similar effects were noted when tetanic and test reflexes were elicited by stimulation of separate nerves.

There is every indication that Woolsey and Larrabee (38) must have observed post-tetanic potentiation, but their mention of facilitation in response to test shocks stimulating roots adjacent to that tetanized suggests the participation of additional mechanisms that apparently have not operated in the circumstances of the present experiments. Prolonged positivity, the basis for increase in afferent spike size, in their experience was confined to the root tetanized.

Striking post-tetanic effects were obtained by Bremer and Kleyntjens in

their study of the spinal cord of batrachians (5). Strongly suggested by their records is the conclusion that the post-tetanic facilitation of which they speak is related primarily not to a potentiating mechanism, but to after-discharge. It may be noted that after-discharge is a prominent feature in the spinal control of certain batrachian muscles (3–6), including iliofibularis, the contractions of which provided the usual indicator in the experiments of Bremer and Kleyntjens. Although future experiment may prove otherwise, present indications are that the phenomenon of Bremer and Kleyntjens differs fundamentally from post-tetanic potentiation as manifested by the monosynaptic reflex paths of the cat spinal cord.

Potentiation at Other Junctions.—More than perfunctory mention of potentiation at other junctions cannot be justified in the absence of experimental observation. It does seem possible, however, that the operation of positive after-potential in the motor nerve fibers might account for post-tetanic decurarization (2) and potentiation of end-plate potential (13), but not, for instance, for potentiation in normal muscle, which Brown and von Euler (10) clearly distinguished from decurarization.

SUMMARY

Following tetanic afferent stimulation of a monosynaptic reflex pathway, the transmission through that pathway of isolated reflex volleys is enhanced for some minutes. Post-tetanic potentiation is comparable in the monosynaptic reflex arcs of flexor and extensor muscles. The facilitator and inhibitor actions of monosynaptic reflex afferent fibers, as well as the transmitter action, are potentiated following tetanization. Little post-tetanic change attends reflex transmission through plurisynaptic reflex arcs.

Various tests for excitability change made independently of the tetanized afferent fibers reveal none or a slight depression. Hence the potentiating influence of a tetanus is limited to subsequent action on the part of the recently tetanized fibers themselves. Increase in the size of the individual impulses comprising an afferent volley such as might occur during positive after-potential, would accommodate the requirement for a limited process and provide for increased synaptic action. The proposed association between post-tetanic potentiation and positive after-potential (*i.e.* hyperpolarization) is supported by the following lines of evidence:—

1. Changes in intensity and duration of potentiation with change in frequency and duration of tetanic stimulation are characteristic of, and parallel to, the changes of positive after-potential in similar circumstances.

2. Afferent impulses are increased following a tetanus, and in a fashion that parallels the course of monosynaptic reflex potentiation.

Post-tetanic potentiation, as here described, and after-discharge, whatever may be its mechanism, are unrelated phenomena.

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DORSAL COLUMN CONDUCTION OF GROUP I MUSCLE AFFERENT IMPULSES AND THEIR RELAY THROUGH CLARKE'S COLUMN*

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Presented at this time are the results of some experiments designed to explore the intramedullary projections of certain primary afferent neurons, and the manner of their synaptic articulation with secondary afferent neurons. Attention has been focussed upon the study of impulses engendered in the large $(22-13\mu)$ or so-called Group I afferent fibers that arise in muscle. Some observations upon impulses initiated in the larger cutaneous afferent fibers are included too, for in concurrent examination of the two sorts of afferent impulses is revealed some difference in the intramedullary arrangement of the primary projections from muscular and cutaneous afferent neurons. The cat has been used for experiment, either as a decapitate preparation, or narcotized by means of dial.

Appearance, in ascending projection, of a Group I afferent volley. Figure 1 illustrates dorsal column activity consequent to stimulation of nerves that supply the hamstring flexors, semitendinosus and biceps femoris posterior. To obtain the records of Figure 1 strength of stimulation was adjusted so that a triphasic spike potential of simple contour resulted at the root entry zone (Fig. 1A). This spike potential represents the conducted action of Group I afferent fibers. For leading, electrodes were placed one upon the cord dorsum, the other at a distance on non-nervous tissue. Record A of Figure 1 was obtained with the "cord" electrode situated at the junction of seventh lumbar and first sacral segments. The ensuing records, B–P, were made each following a rostral shift of 5 mm. in the location of the "cord" electrode. Thus, in effect, a volley of impulses is traced, in Figure 1, for a distance of 7 cm., from its entry into the dorsal column to the level beyond which it could no longer be identified.

As a Group I afferent volley ascends the dorsal column, it is seen that amplitude of the recorded spike potential decreases and that conduction velocity is slowed in a manner much like that described by Gasser and Graham (2) when observing intramedullary conduction of dorsal root volleys. It is notable that neither conduction velocity nor recorded amplitude decreases in a regularly

* A report of the observations herein described was presented before the American Physiological Society at its Atlantic City Meeting, 1948 (9).

progressive manner. But details of this sort are seen to better advantage in graphical representations constructed by plotting recorded amplitude and response latency as functions of conduction distance.

To be found in Figure 2 are graphs drawn, in the manner indicated, to represent the experimental findings concerning conduction of a Group I afferent volley arising peripherally in the flexor hamstring nerves. The scale of ordinates at the left of Figure 2 measures conduction latency, in msec., from the stimulation site on the peripheral nerves to the recording station on the dorsal col-
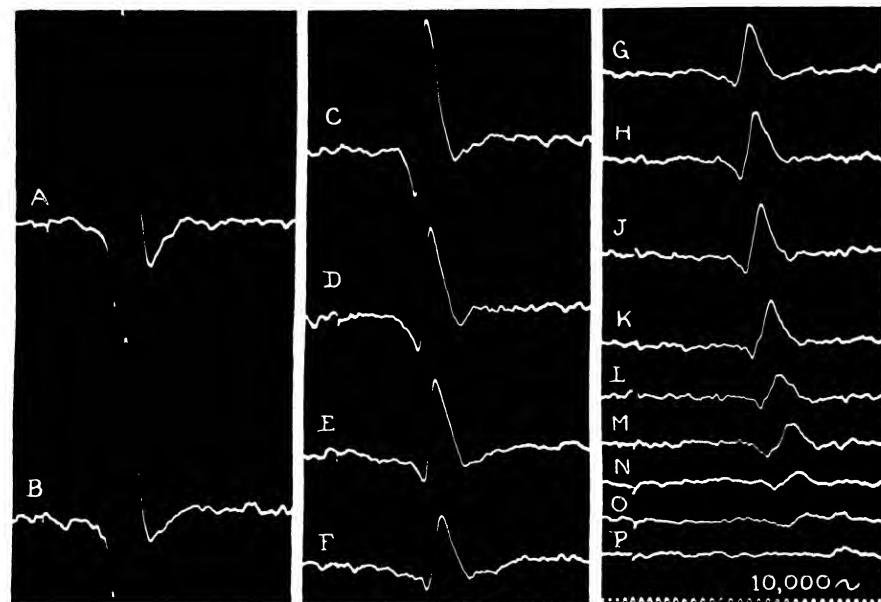
FIG. 1. Conduction in intramedullary projections of semitendinosus-biceps femoris posterior Group I afferent neurons, recorded in successive 5-mm. steps for a distance of 7 cm. from a point at root entry zone.

umn. Amplitude of the spike potential response is represented in per cent of maximum on the scale of ordinates to the right of Figure 2. Appearing on the scale of abscissae is total conduction distance between stimulating and recording leads. Zero on the scale of abscissae is determined by the location, on the specified peripheral nerve, of the stimulating cathode. A secondary abscissa indicates the segmental boundaries established according to the convention that the most rostral rootlet from a given segmental nerve-root marks the rostral limit of the segment. For each electrode placement a dot represents the measured shock-response interval, a circle the recorded spike potential amplitude.

Most prominent feature in the curve of amplitude displayed in Figure 2 is the stepwise fashion in which decrease of the spike potential takes place. Amplitude is maximal at some point within the segments that receive fibers from the stimulated muscle nerve (L_7, S_1, and to some extent L_6 in the case of the flexor hamstrings). A major discontinuity is to be found in the lower third lumbar segment.

Conduction velocity of the Group I afferent volleys is reasonably represented, as in Figure 2, by a series of linear plots of progressively changing slope. Throughout the entry region of active fibers into dorsal column the characteristically high nerve-root velocity of a Group I afferent volley (111 M
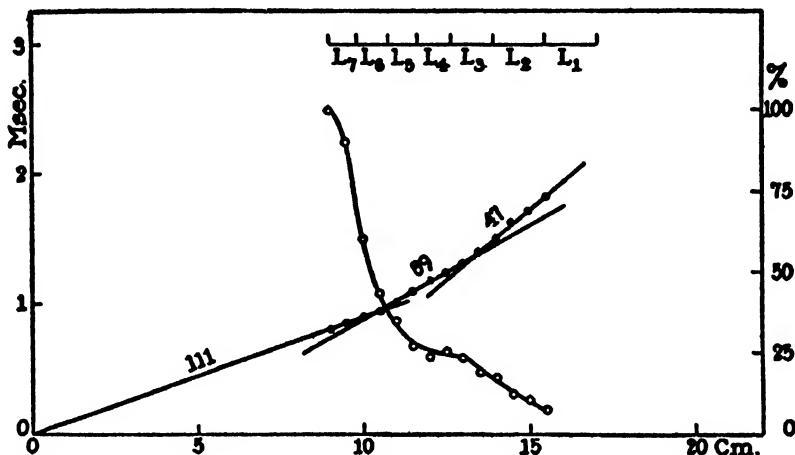
FIG. 2. Intramedullary conduction of flexor hamstring Group I volley. Circles: spike potential amplitude. Dots: conduction latency. Conduction velocity given in M. per sec. for each slope

per sec. in this instance) is maintained. In the sixth lumbar segment velocity drops, seemingly quite suddenly, to a new value approximately two-thirds that in the extramedullary fibers (69 M. per sec.). A second decrease in velocity (to 47 M. per sec.) is evident after the volleys reach the third lumbar segment. In the experiment illustrated in Figure 2 activity of Group I fibers was not found beyond the lower limit of the first lumbar segment. When, as in other experiments, activity can be traced into the lower thoracic region, a third drop in conduction velocity is found to occur in the first lumbar segment. An instance of the latter finding is presented in Figure 3.

The nerve to quadriceps, by virtue of its large size and number of contained Group I afferent fibers (8) is an excellent source for Group I afferent volleys. Figure 3 illustrates the intramedullary conduction of such volleys engendered in the quadriceps nerve. The manner of plotting is that employed for Figure 2.

Due to the different segmental representation of the two muscle groups, the recorded amplitude of volleys initiated in quadriceps nerve is maximal in the fifth lumbar segment, two segments rostral to the point of maximum for the flexor hamstring volleys plotted in Figure 2. Decrease in amplitude of the response with conduction is precipitous, there being, however, a recognizable discontinuity near the upper border of the fourth lumbar segment. Rostral to the second lumbar segment the response was identifiable in the original records, and its conduction latency was measureable in reasonable approximation, but
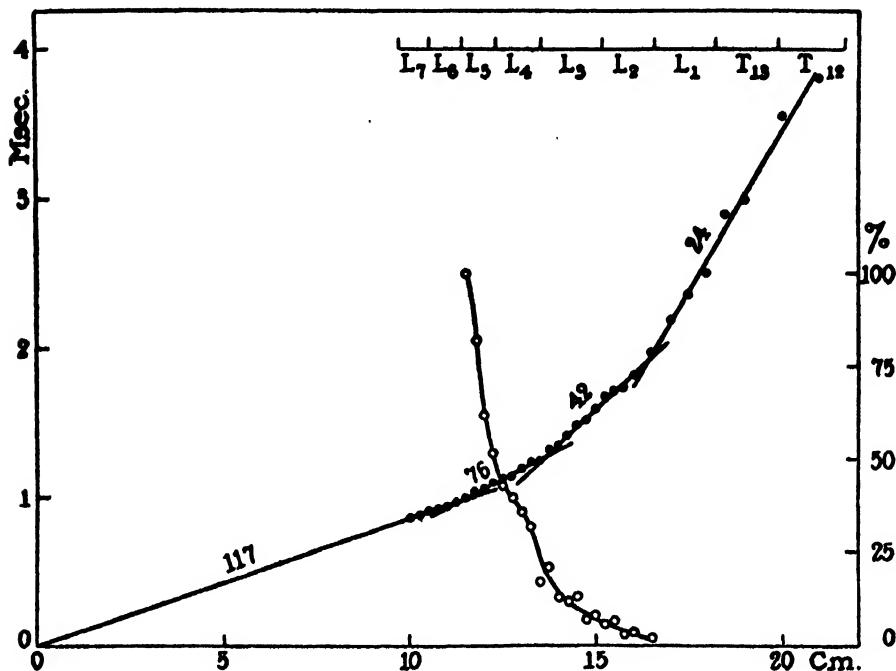
FIG. 3. Intramedullary conduction of quadriceps Group I volley. Otherwise as in Fig. 2.

it would be fruitless to attempt representation of response amplitude, for the electrical disturbance involved scarcely exceeded the background activity.

Conduction velocity in the dorsal column of the quadriceps Group I afferent volleys may be represented as in Figure 3 by a succession of linear plots. Initial nerve-root-cord conduction velocity is 117 M. per sec., a value in keeping with the fact that quadriceps nerve contains the largest afferent fibers (8). Within the fifth lumbar segment velocity drops to 76 M. per sec., or about two-thirds the initial value. At the junction of third and fourth lumbar segments the second drop in velocity to about one-third (42 M. per sec.) takes place. As activity

penetrates into the first lumbar and lower thoracic segments, the small size of the responses, as already noted, lessens the accuracy of latency measurements. However, a slope of 24 M. per sec. reasonably represents conduction velocity therein, and demonstrates the occurrence, at the first lumbar segment, of a third drop in velocity.

Intramedullary conduction of muscle Group I and of cutaneous afferent volleys contrasted. Intramedullary conduction of cutaneous afferent volleys is distinctly different from that of muscle Group I afferent volleys. Concerning the amount of activity present at a given level, following stimulation of a representative cutaneous nerve, very little can be said, for the intramedullary spike potential is relatively small at best (for instance, see Ref. 7, Fig. 5), and it is followed by a large negative intermediary potential. Considerable temporal dispersion attends conduction, in the dorsal column, of a cutaneous nerve volley, with the result that intramedullary spike potential and negative intermediary potential rapidly become inextricably intermingled. Adding to the confusion there is to be found the dorsal column relay discharge described by Hursh (5). In the circumstances outlined only a very general statement on intramedullary conduction of cutaneous nerve impulses is warranted.

As a volley of cutaneous origin ascends the dorsal column there is considerable decrease in amplitude and increase in dispersion of the recorded response until the anterior limit of the lumbar enlargement is attained, beyond which—for a good distance—little further change can be detected. It is possible with reasonable precision to measure the response latency, at different levels, of the impulses that contribute to the forefront of the recorded action, and the information so gained may be represented graphically. It is well to remember, however, in view of the inhomogeneity evidenced by great dispersion, that the activity of some fibers, portrayed graphically, cannot be taken as representative of the group as a whole.

In Figure 4 intramedullary impulse conduction by muscle and cutaneous afferent fibers is contrasted. To provide a cutaneous nerve volley the peroneal cutaneous nerve was chosen. Parallel observations on conduction in muscle Group I afferent fibers were made employing, for stimulation, nerves to the pretibial muscles, tibialis anterior and extensor longus digitorum. This choice of nerves permits an arrangement for equal peripheral conduction distance.

As plotted in Figure 4, nerve-root velocity of the pretibial muscle Group I afferent volleys is 99 M. per sec., that of the peroneal cutaneous nerve volleys 78 M. per sec. At first sight the former value might appear to be unduly low. It should be noted, however, that the largest Group I afferent fibers from pretibial muscles are of lesser diameter than those from most other hind-limb muscles. Indeed, the variation in nerve conduction velocities encountered in Figures 2, 3, 4 and 5 is just that to be expected from comparative study of the afferent fibers in the nerves concerned (8).

With equal peripheral conduction distances, the pretibial muscle nerve Group I volleys, at the spinal cord, lead the peroneal cutaneous nerve volleys by just under 0.5 msec. In the seventh and sixth lumbar segments nerve-root velocities prevail apparently for both muscular and cutaneous afferent volleys. Above the sixth lumbar segment velocity of the muscle Group I afferent volleys drops to 52 M. per sec., and again, at the upper margin of the third lumbar segment, to 27 M. per sec. Group I afferent volleys, in this experimet, disappear in the first lumbar segment. In contrast the cutaneous nerve volleys in the course of intramedullary conduction through the lumbar cord suffer rela-
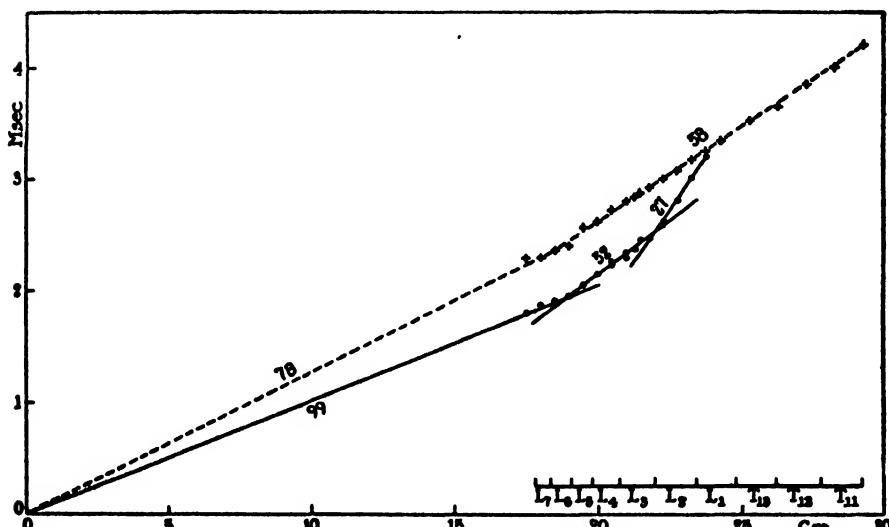
FIG. 4. Conduction velocity in the intramedullary projections of Group I fibers from pretibial muscles and of the larger fibers of peroneal cutaneous nerve compared.

tively less decrease in conduction velocity (from 78 to 58 M. per sec.), and continue thereafter without further loss in velocity until the cervical enlargement is reached.

An interesting consequence of the differences in conduction properties of the muscle and cutaneous afferent fibers is that the first impulses of a volley initiated in a mixed peripheral nerve, such as the tibial or peroneal nerves, will, in the nerve, dorsal roots and lower lumbar dorsal column, be contributed by fibers of muscle Group I origin, and in the upper lumbar dorsal column, and further rostrally, by fibers of cutaneous origin (cf. also Fig. 5).

Analysis by use of antidromic volleys. Study of antidromic conduction in primary afferent neurons—that is, by the recording on a selected peripheral nerve of volleys initiated in the dorsal column—presents certain advantages

and disadvantages not attaching to the observation of orthodromically conducted activity. The two approaches thus are suitably complementary.

Figure 5 illustrates the result of an experiment performed in examination, by the antidromic method, of dorsal column conduction velocities. Impulses were recorded in the nerve to gastrocnemius (dots), and in the sural nerve (crosses), with the electrodes so arranged that conduction distances in the two nerves were equal. Each point on the plots represents a shock-response interval; it is located on the ordinates according to the duration of that interval, and on the abscissae according to the location in the dorsal column of the stimu-
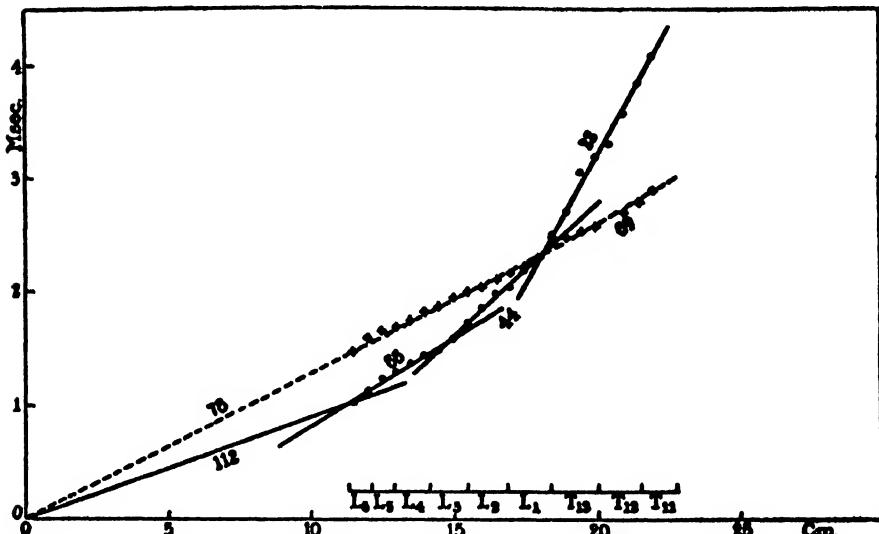
FIG. 5. Intramedullary conduction velocity of gastrocnemius Group I fibers and of the larger sural cutaneous afferent fibers compared from experiment employing antidromic conduction.

lating electrode pair. The principal abscissa expresses in cm. the distance between recording and stimulating electrodes; a secondary abscissa indicates the segmental boundaries. By this method of plotting, the course of antidromic conduction may be compared directly with that of orthodromic conduction.

Comparison of Figure 5 with Figures 2, 3, and 4 shows that the experimental result concerning intramedullary conduction velocities is the same whether impulses are initiated orthodromically or antidromically. In Figure 5 Group I impulse velocity in the gastrocnemius nerve, and in the roots, averages 112 M. per sec. Between the seventh and third lumbar segments velocity is 66 M. per sec. Between the third and first lumbar segments velocity is 44 M. per sec., while in thoracic segments represented velocity is only 22 M. per sec. In con-

trast, loss of velocity by the foremost cutaneous afferent impulses is not great. In amplitude the changes in Group I volleys with intramedullary conduction distance revealed by the antidromic method in no way resemble those suffered by orthodromic volleys. The reasons for this are various and complex; a discussion of them follows interpretation of the velocity changes that have been described.

Concerning velocity changes in central projections of afferent fibers, particularly Group I. For purposes of discussion it will be assumed that conduction velocity in spinal tracts, as in peripheral nerve, is a function of fiber diameter. While there is no rigorous proof of the function, there are sufficient scattered observations to suggest that it is not unreasonable to treat of central tract fibers according to the diameter-velocity rule for peripheral myelinated fibers (3). This being so, decrease in impulse velocity as a volley ascends the dorsal column is taken as evidence that all the responding fibers rostral to the point of velocity change are of lesser diameter than some caudal to that point. An accounting for the decrease in diameter could be made along any one of or combination of the following three lines: (i) *Segregation*, by which is implied a failure of the larger fibers to project as far rostrally as do the smaller fibers; (ii) *Tapering;* and (iii) *Branching*, with the daughter fibers assuming a lesser diameter than that of their parent fibers.

(i) While it is obvious that segregation of fibers occurs in the dorsal column, the velocity changes encountered by a Group I volley cannot depend in significant degree upon this factor since, in observation of antidromic conduction, it is found that volleys initiated in the upper lumbar dorsal column and volleys initiated near the root entry zone have, in a selected muscle nerve, comparable and characteristically high conduction velocities, despite differences in conduction velocity during their intramedullary course.

(ii) For the purposes of the present analysis it would appear justifiable to neglect the possibility that tapering may contribute to decreasing conduction velocity, the more so since velocity changes seem to occur with a certain abruptness.

(iii) In every experiment the initial fall in velocity of a Group I afferent volley has occurred at the rostral limit of segmental representation of the stimulated muscle nerve. Otherwise stated, the initial fall in velocity occurs at the region wherein the entering dorsal root fibers undergo their initial division to form ascending and descending fibers of the dorsal columns, and reflex collaterals. It seems certain, then, that the initial decrease of conduction velocity may be referred to the initial branching of the afferent fibers.

The second decrease in velocity of Group I afferent volleys uniformly has been seen to occur in, or immediately adjacent to, the third lumbar segment. The fact demonstrated below that Group I fibers within this region of the cord form synaptic articulations with the cells of Clarke's column provides the evi-

dence for stating that the second velocity drop encountered by ascending Group I volleys, like the first, is the aftermath of fiber branching, the collaterals formed in the process being directed to Clarke's column.

It is presumed that the third velocity drop suffered by ascending Group I volleys is susceptible of similar explanation.

On significance of amplitude changes in orthodromic and antidromic Group I volleys. In Figure 6 are plotted curves relating amplitude of Group I volleys to conduction distance. Curves A and B of Figure 6 were constructed from observations made with the use of a single preparation: In one instance (curve A) orthodromic volleys, initiated in the muscle nerve, were recorded at various levels of the dorsal column, in the other (curve B) antidromic volleys, initiated at various levels in the dorsal column, were recorded at a single station on the muscle nerve. For each curve amplitude is expressed on the ordinate in per cent of the maximum, and conduction distance in cm. rostrally from a point in the sixth lumbar segment is expressed on the abscissa. As in previous figures a secondary abscissa indicates the segmental boundaries. Inspection reveals the curves of Figure 6 to be strikingly different.

Amplitude of a Group I response, whether recorded in the dorsal column after orthodromic conduction, or in the peripheral nerve after antidromic conduction, will depend upon a number of variables: (i) the number of Group I fibers stimulated, (ii) the number of those fibers that extend from the peripheral nerve to any given level of the spinal cord, (iii) dispersion, (iv) spike size per fiber, and (v) the amount of active tissue. Of these factors, the number of Group I fibers stimulated does not enter into the qualitative differences in the course of amplitude change now to be discussed. Likewise, it is obvious that the number of Group I fibers that extend from the peripheral nerve to a given level of the cord is the same regardless of the direction in which they are made to carry impulses. Progressive decrease in the number of Group I afferent fibers represented in the dorsal column obviously is a factor contributing to the descent of the curves, but in no way can it account for differences between them.

Dispersion certainly contributes to the descent of the curves as plotted. Indeed, differences between the curves in some small degree can be ascribed to the influence of dispersion for—as is well known—impulses recorded in the triphasic form are soon lost to view by reason of temporal scatter. However, as Figure 1 demonstrates, dispersion of a Group I volley throughout most of the lumbar cord is not great.

The fourth-named factor, spike size per fiber, presumably could contribute toward difference between curves A and B of Figure 6; to what extent, however, is problematical. Decrease in diameter of the Group I intramedullary projection fibers being, at the present time, the only reasonable basis for the observed slowing of Group I volleys as they ascend the cord, the question of parallel decrease in spike height per fiber naturally arises. While this would

influence progressively the size of an ascending projection volley, it would not similarly affect antidromic volleys recorded peripherally. While this influence would work in the direction of the observed disparity, its importance cannot be assessed independently.
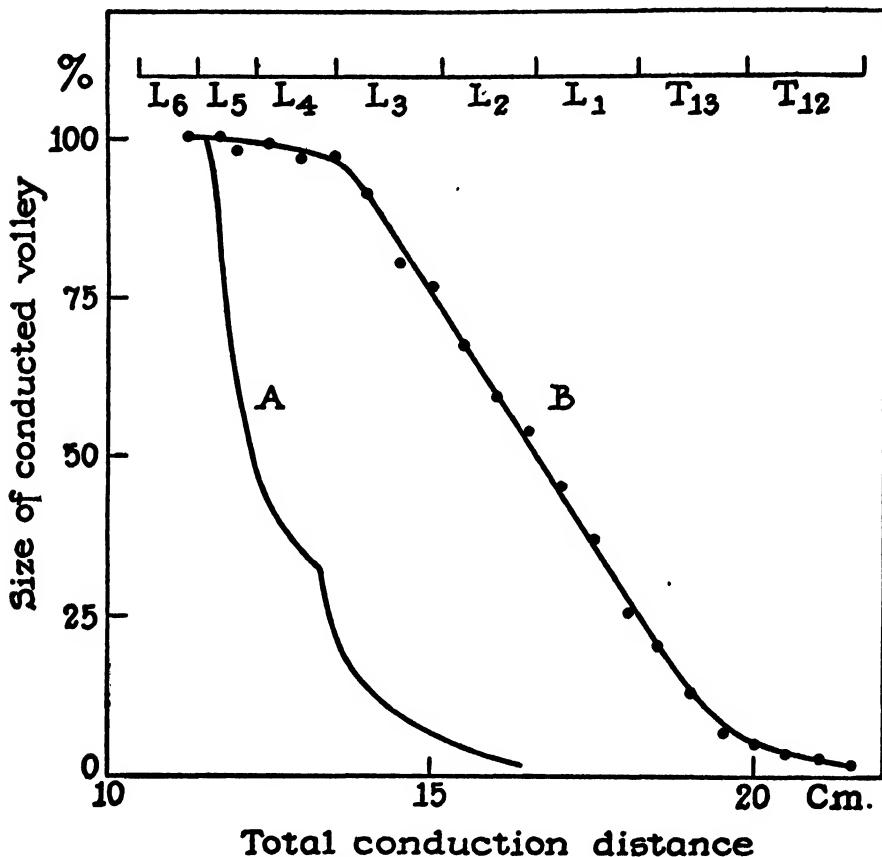
FIG. 6. Amplitude changes with conduction distance of quadriceps Group I afferent fiber volleys. Same preparation as for Fig. 3. Curve A: amplitude changes of orthodromic volleys as detailed in Fig. 3. Curve B: amplitude changes of antidromic volleys. For reasons discussed in text curve B yields more valid approximation to an estimate of relative number of Group I fibers extending from the selected muscle nerve to any given level of spinal cord.

Undoubtedly the last-named factor, amount of active tissue, is the prime determinant of the differences between curves A and B of Figure 6. A Group I afferent orthodromic volley entering the spinal cord immediately occupies not only the parent intramedullary segments, but also the longitudinal fibers of

the initial dichotomy, and a heavy concentration of collaterals—all together comprising a considerable expansion of the amount of active tissue. It is at a point in this region that the orthodromic intramedullary spike potential is maximal. Above the segmental limit of entering active fibers there are no more contributing parent fibers and the number of collaterals becomes progressively less. Stated otherwise, there is a large decrease in the amount of active tissue, involved by a Group I orthodromic volley, that is totally unrelated to the number of Group I fibers contributing the volley. On the contrary, in the peripheral nerve the amount of active tissue, in the first approximation, is related to the number of Group I fibers activated by any given antidromic stimulus to the dorsal column.

To the foregoing considerations should be added another, of an anatomical nature, that would influence the magnitude of orthodromic response in the direction required by Figure 6. In view of the association between Group I afferent fibers and Clarke's column, one must entertain the proposition that the orthodromically conducted spike potential would decrease, all other things being equal, if the bundle of active fibers were at some point to plunge from a region more or less near to the dorsal surface, and hence close to the exploring surface electrode, into the deep recess of the intercornual portion of the dorsal column to place a layer of inactive tissue between the bundle of conducting fibers and the searching electrode. The appearance, following lesions of the lower cord or roots, of ascending degeneration that shifts not only medially with rostral progression, as is widely stated, but also, on extending through the lower half of the thoracic region, shifts dorsally away from the gray matter suggests that Group I fibers terminating in Clarke's column occupy the deep position. Particularly instructive in this connection is Experiment III, and Figures 11–16 from Sherrington's study on ascending degeneration (10) in which the degeneration in the dorsal column, following transection in the lower thoracic cord, is seen to split into deep and superficial zones, the former soon disappearing while the latter continues upwards always avoiding the gray matter.

A major purpose of the foregoing discussion has been to arrive at an interpretation by which it may be possible to estimate, however crudely, the extent to which Group I fibers project rostrally through the dorsal columns. Such an estimate clearly must be based upon the evidence, as in curve B of Figure 6, from antidromic conduction. For instance, it is apparent from comparison of curves A and B of Figure 6 that the initial, major loss of amplitude suffered by Group I volleys in orthodromic recording takes place without appreciable defection of Group I fibers from the ascending projection system. The discontinuity in curve A is situated at a spinal level beyond which, according to the interpretation of curve B, Group I fibers begin to terminate, so that decrease in the number of active Group I fibers, by reason of termination, is added to

the other factors in determining the second step in the loss of amplitude suffered by orthodromic Group I volleys. Thus it is seen that amplitude change with conduction of an orthodromic Group I volley ascending the dorsal column is the product of complex circumstances, and admits of no simple interpretation.

Estimation of the number of Group I fibers reaching from a given muscle nerve to a given cord level at first sight might seem not to be a difficult task. It has been shown that the approach by means of orthodromic recording is useless, and in consequence reliance must be placed in the antidromic method. It is well to be aware, however, of the faults inherent in the latter approach. In the first place, when stimulating the dorsal columns one no longer has the threshold relations of nerve fibers to aid in selective stimulation. Secondly, a stimulus to the whole dorsal column evokes a tremendous volley of impulses therein, which, although possibly not involving any of the fibers under observation, might, after some variable conduction distance, serve as an electrical stimulus to such fibers. While ordinarily the law of isolated conduction holds unless some special appropriate conditions are established to support "ephaptic" transmission (1), the obvious fact, in antidromic stimulations, that the terminal regions of many Group I fibers are subjected to the external fields of large volleys certainly does introduce special, and possibly appropriate, conditions for cross stimulation. That afferent fibers are subject to stimulation centrally of course is obvious from the fact of the dorsal root reflex (12).

Mindful of the foregoing difficulties and the pitfalls they present, it still follows that the amplitude curve (6B) obtained by the use of antidromic volleys is the nearest practical approach to a measure of the longitudinal extent of the Group I afferent fiber intramedullary projection. From experiments such as that exemplified by Figure 6B it seems a fair conclusion that substantially all the Group I afferent fibers from a given hind-limb muscle, after supplying direct collaterals to the appropriate motoneurons (6, 7), and possibly to other systems as yet unidentified, attain the level of the inferior end of Clarke's column, and that substantially all those fibers terminate within the longitudinal limits of Clarke's column. One will appreciate the fact that this manner of termination of itself does not prove the relation of all or any Group I fibers with Clarke's column. A direct examination of the relation follows.

Some properties of synaptic transmission in Clarke's column. The distinctive manner of impulse conduction by the ascending projections of Group I fibers formed the original basis for supposing that an important function of those projections might be the relay of activity, through synaptic connections with the neurons of Clarke's column, to the dorsolateral ascending tract of Flechsig. This suggestion furthermore seemed not unlikely since, in the experience of Grundfest and Campbell (4), it was only when muscle afferent fibers were present in a stimulated peripheral nerve that the activity recorded in the region of Flechsig's tract was attributable to tract rather than propriospinal fibers. It

remains then to demonstrate, by experimental test, the proposed relation between Group I afferent fibers and Clarke's column. In the first instance the method that has been employed is that by which Group I fibers were shown to constitute the afferent limb of the monosynaptic reflex pathways (6).

Seen in its fully developed form as evoked by a single dorsal root shock, response of the dorso-lateral tract consists of several more or less discrete discharges in rapid succession that degenerate progressively into a diffuse, dispersed and random discharge. The general features of the tract response are recorded in Figures 7 and 8. To obtain observations of the tract response satisfactory for measurement a lead was placed on the cord surface at the dorso-
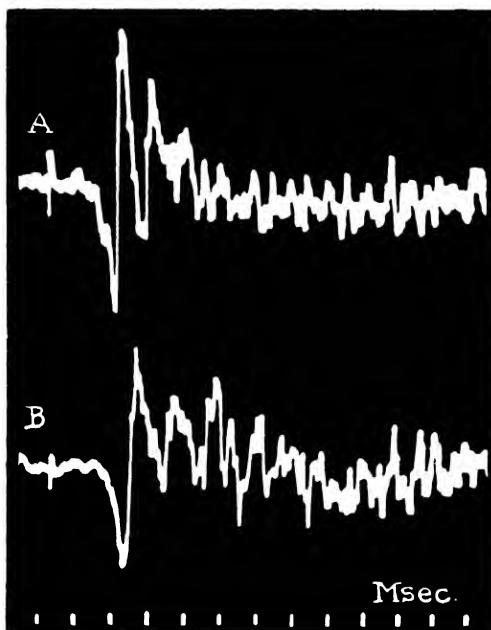
Fig. 7 Responses of dorso-lateral tract to single stimulations of combined seventh lumbar and first sacral dorsal roots.

lateral angle of the lateral white column and pitted against a distant lead located on non-neural tissue. By this means, made possible by the favorable location of the tract, the inevitable damage from needle prodding and consequent distortion of the electrical activity can be obviated.

Records A to G of Figure 8 were obtained from the dorso-lateral tract by the means indicated, following stimulation with different shock strengths of the seventh lumbar dorsal root. The "cord" electrode was located in the ninth thoracic segment 14 mm. rostral to a section, in the tenth thoracic segment, of

the dorsal column. To the right, below each recording in Figure 8, is to be found a figure expressing in per cent of maximum the measured size of the afferent volley employed to evoke the recorded response.

Inspection of Figure 8 shows that the tract discharge is well developed when the causal dorsal root volley is but two per cent of maximum (record 8A). As the afferent volley is increased the tract response grows rapidly (records 8B and C) until, with an afferent volley only 48 per cent of maximum, the tract response reaches a ceiling (record 8D), there being no further increase with continued increase in afferent volleys (records 8E, F and G). Thus the development of discharge in the dorso-lateral tract with incremental, graded afferent
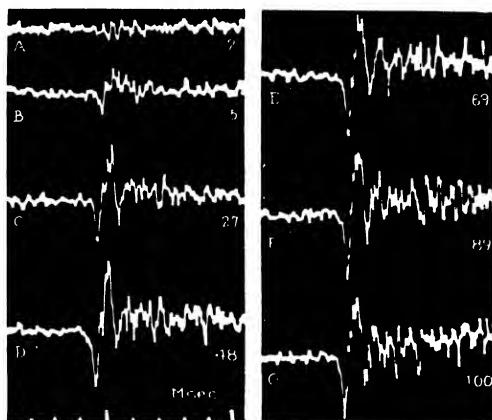
FIG. 8. Change in response of dorso-lateral tract with change in strength of presynaptic stimulation. Measured size of afferent volley evoking each recorded response is represented numerically in per cent of maximum to right of each record.

stimulation closely parallels the growth of discharge in similar circumstances through the monosynaptic reflex pathways (6). From such evidence it is concluded that the same band of afferent fibers is responsible for postsynaptic action in the two systems. The delayed tract discharges (unlike the multineuron reflex discharges which continue to develop with increase to maximum of the afferent volleys) are, like the initial tract discharge, seemingly fully developed when evoked by afferent volleys somewhat less than 50 per cent of full magnitude. According to this evidence the Group I fibers alone are concerned with the origination of activity in Flechsig's tract.

Graphical representation of the relationship between size of afferent volley and size of tract discharge resulting from it possesses certain advantages. Included in Figure 9 are the results of three experiments of the sort illustrated by Figure 8. Each experimental point is located on the abscissae according to the size of evoking afferent volleys in per cent of maximum, and on the ordinates

according to the average size, from a number of repetitions of the observation, of the initial tract discharge evoked by a given afferent volley. Observations from the several experiments are identified by suitable distinguishing symbols. For comparison with the plot of experimental observations is to be found a curve similarly constructed, but for which monosynaptic reflex responses evoked by graded dorsal root shocks are related in magnitude to the size of the evoking afferent volleys. Coincidence of the two curves at the maximum, with the curve for Flechsig responses always, on the abscissa, to the left of the curve for the monosynaptic reflex, demonstrates that the Group I band of afferent fibers, in
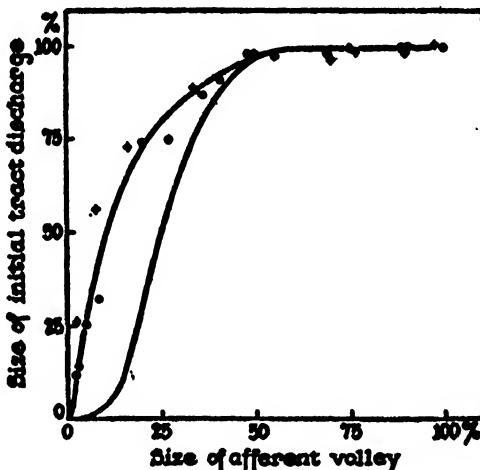
FIG. 9. Size of tract response as function of causal afferent volley size illustrated from three experiments, experimental points of which are distinguished by different symbols. For comparison is a curve relating monosynaptic myotatic reflex discharge to size of afferent volley.

addition to its role in the execution of myotatic reflexes, constitutes the afferent channel to Clarke's column.

Most interesting detail of the curve in Figure 9 for response in the dorso-lateral tract, and the only essential manner in which the relay from Group I afferent fibers to the cerebellar system differs from the monosynaptic relay of the myotatic reflex system, is its abrupt rise from the origin as contrasted with the markedly sigmoid curve for incrementing response of the motoneurons. Indicated by this difference is an extremely powerful synaptic articulation that causes an afferent volley, however weak it may be, to secure a postsynaptic response in the secondary neurons of the ascending tract, whereas it is only after considerable summation at the motoneuron pool that reflex discharge takes place.

It is apparent now that the Group I afferent fibers provide a unique example

of fibers, by dichotomy, coming into direct synaptic connection both with moto-neurons and with afferent tract neurons, and it is apparent further, from the observation illustrated by Figure 9, that this single group of nerve fibers pos-sesses at the one location terminations of one functional sort and at the other location terminations of another functional sort. The type of relay found in Clarke's column is known to exist elsewhere among the afferent systems, in evidence of which one recalls the description by Therman (11) of an essentially one-to-one relay in the cuneate nucleus between dorsal column and lemniscus. Indeed, there are some grounds for supposing that this type of articulation may be rather generally distributed among the relay points of the afferent paths in the central nervous system. On the other hand, it is quite obvious that no motor path is so equipped to dominate the motoneurons.

Activity arising at the periphery among the Group I afferent fibers may be traced throughout its course, first in the spinal roots and dorsal column (as in Figures 1–5), and then, following synaptic relay, along the lateral column for the remaining length of the spinal cord, into the restiform body and finally at the cerebellar cortex. Germane to the present discussion is a consideration of the temporal course of such activity in the spinal cord, with particular reference to the delay introduced by reason of synaptic transmission and to the velocity of postsynaptic conduction. These events may be discussed in connection with Figure 10.

In order to secure a postsynaptic discharge of suitable proportions during the experiment illustrated by Figure 10, the nerves of flexor longus digitorum plantaris and gastrocnemius (with soleus) were combined for afferent stimula-tion, the shocks employed being of a strength designed to limit stimulation as far as possible to the Group I band of fibers. By this selection of nerves a large fraction of the muscle afferent inflow of the tibial nerve is obtained without the heavy cutaneous, and joint, contributions. In Figure 10 the dorsal column responses evoked by stimulation of the nerves named are represented by dots in the manner initially discussed in connection with Figure 2. Additionally, responses recorded at selected stations along the dorso-lateral tract are repre-sented, by circles, in their proper relations of time and total conduction distance. Only the conduction latency for the initial tract volley, as defined in Figures 7 or 8, is considered in the graphical construction.

Detailed description of the nerve-root-dorsal column conduction of the pre-synaptic Group I volleys is not necessary, for the pattern of other experiments is followed closely. Initial velocity of these volleys is 104 M. per sec., and de-creases take place, in the sixth lumbar segment to 68 M. per sec., and in the third lumbar segment to 30 M. per sec. After a time delay in the mid-lumbar cord, occasioned in part by slowing of the presynaptic impulses and in part by

synaptic delay, the postsynaptic impulses are recorded as travelling at a uniform velocity of 109 M. per sec.

If, as in Figure 10, the plot of tract conduction be extended caudally (in the first instance without regard to the segmental origin of the tract discharge), it is possible to measure at any level of the lumbo-sacral cord the temporal displacement between the slopes for presynaptic and postsynaptic conduction. At no point, even at the lowest level of afferent fiber entry from the stimulated muscle nerves, is that temporal displacement greater than 0.75 msec. It follows then that the initial tract discharge is the product of monosynaptic transmission from primary afferent neurons to secondary tract neurons. Concerning relay of
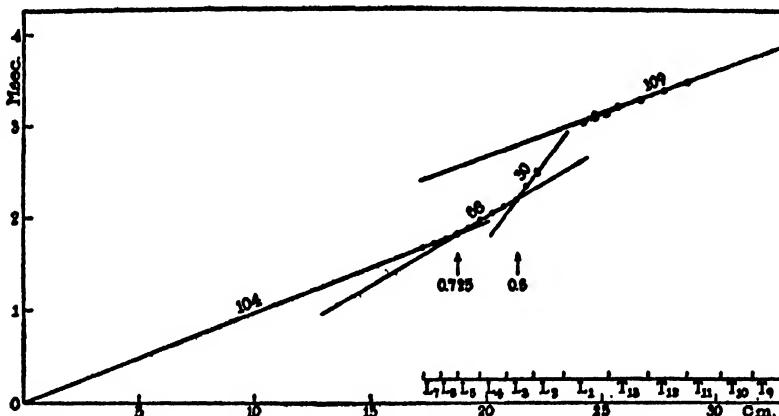
FIG. 10. Presynaptic and postsynaptic conduction velocities in myotatic (Group I) afferent fiber-dorso-lateral tract projection system. Dots: presynaptic conduction. Circles: postsynaptic conduction. Figures below arrows give temporal displacement between presynaptic and postsynaptic volleys at levels indicated by arrows.

the later discharges virtually nothing can be said. Within the framework of the then current thinking, Grundfest and Campbell (4) found it necessary to suppose the existence of internuncial chains intercalated in the pathways to the dorso-lateral tract, but the need for invoking internuncial circuits perhaps now is not so urgent. Interpretation, therefore, might better be left until more is known of the fundamental properties of synapses in afferent systems.

Figure 10 is suggestive concerning the level at which the initial tract discharge is elaborated. On the supposition that a "minimum" synaptic delay of 0.5–0.6 msec. must be allowed for monosynaptic transmission in the present circumstances of latency measurement between presynaptic and postsynaptic volleys, it follows that the site of transmission is located probably at and caudal to the second inflection point in the velocity plot of the primary afferent impulses or, in other words, at what presumably is the caudal end of Clarke's column.

SUMMARY

The largest afferent fibers, serving muscle, for the most part terminate in the upper lumbar and lower thoracic segments. During this relatively truncated course in the dorsal columns, after supplying myotatic reflex collaterals to the motoneurons, these fibers came into relation with the cells of C ke's column where a relay is established for the projection to the cerebellum of stretch-evoked activity.

Conduction velocity of impulses in the primary afferent fibers is strikingly non-linear. A volley engendered in the large afferent fibers of muscle has an initial velocity approximating 110 M. per sec. Once these fibers have entered the spinal cord velocity drops to about two-thirds the initial value. Further changes, occurring typically in the third and first lumbar segments, bring velocity to less that one-quarter the initial value. Less spectacular changes attend impulse conduction by the larger cutaneous afferent fibers. Impulses in the dorso-lateral tract conduct at a uniform velocity that compares with the initial velocity of impulses in the presynaptic afferent fibers from muscle.

The synaptic articulations which the large afferent fibers form with motoneurons and with the cells of Clarke's column differ characteristically, considerable summation being requisite for transmission in the motor nuclei, but not in Clarke's column.

Postsynaptic response in the dorso-lateral tract begins with a discharge relayed, with little time delay, at the caudal end of Clarke's column. Following this monosynaptic discharge is a series of discharges occurring with progressively increasing randomness. The mechanism of the later discharges is not known.

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PHAGE FORMATION IN STAPHYLOCOCCUS MUSCAE CULTURES

VI. Nucleic Acid Synthesis during Virus Formation

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Recent papers from this laboratory have described certain general properties of the *Staphylococcus muscae* bacteriophage system. The formation and release of the bacterial virus have been studied in veal infusion medium and Fildes' synthetic medium (1). Cells, with single and multiple infection, in the lag and in the logarithmic phase have been investigated (1, 2). The effect of three nutrient factors has been reported. Two of these factors, one of which is found in various acid-hydrolyzed proteins and the other in yeast, influence the yield of virus liberated per cell in Fildes' synthetic medium (3). The third substance found in yeast accelerates cellular lysis under certain experimental conditions and has no effect on the final yield of virus formed per cell (4). Unpublished observations showed that this *S. muscae* phage is one of the tadpole-shaped bacterial viruses, and that its nucleic acid is primarily, if not all, of the desoxyribonucleic acid type (5).

As a result of these investigations, it is now possible to control to a large extent the formation and release of the phage by this particular strain of *S. muscae*.

In a preliminary study, in which samples were taken early during the course of infection, no effect of the virus was noted on the nucleic acid metabolism of the parasitized host (6). Subsequent experiments, however, have revealed that although the amount of nucleic acid (NA) synthesized by normal and infected cell suspensions is approximately the same, the distribution of nucleic acid between cells and media in normal suspensions was entirely different from the distribution in infected suspensions. Infected cells released ribonucleic and desoxyribonucleic acid into the medium whereas normal cells did not. Infected suspensions have a lower RNA/DNA ratio than normal suspensions.[1] This is due to the fact that infected suspensions synthesize more DNA and less RNA than normal suspensions.

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[1] Ribonucleic acid, desoxyribonucleic acid, will be abbreviated as RNA, DNA, respectively in this paper. NA will be used to indicate RNA plus DNA or total nucleic acid.

EXPERIMENTAL RESULTS

Nucleic Acid Synthesis in Normal Cell Suspensions.—Fig. 1 shows the multiplication of normal cells and the changes in the total amount of RNA and DNA for the total suspension and per 3.0×10^{10} cells. When first taken from the 22 hour slant, the cells contain about as much RNA as DNA. In the 1st hour the RNA per cell increases to 2 times the DNA concentration. As the cells enter the log phase, there is about $2\frac{1}{2}$ times as much RNA synthesized as DNA.
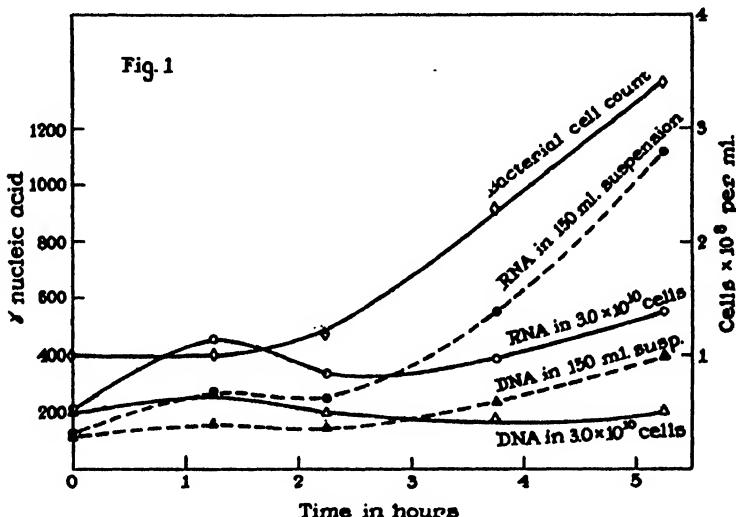
FIG. 1. Nucleic acid changes per cell and for total suspension during normal cellular multiplication.

A 2000 ml. Erlenmeyer flask, containing 800 ml. of synthetic medium, was inoculated with 1.0×10^8 washed cells per ml. and shaken at 37°C. At the times indicated, 150 ml. samples were centrifuged at 3600 R.P.M. (1200 $\times$ g) for 10 minutes, the cells washed in 10 ml. of cold saline, and recentrifuged as above. The cells were then suspended in 4.0 ml. of cold 10 per cent trichloroacetic acid and RNA and DNA determined as in Methods.

After this, the amount of RNA and DNA per cell is fairly constant, although the total RNA and DNA of the suspension increases, as the cells multiply. Sometimes the RNA and DNA concentration per cell reach their peaks in $1\frac{1}{2}$ to 2 hours, instead of 1 hour. When this occurs, it is difficult to interpret the effect of the virus infection on the nucleic acid metabolism of the host, in the cases where the virus is added to the cells after 1 hour, since it is not possible to tell whether the observed increase in nucleic acid synthesis is due to the virus infection. In order to overcome this difficulty it is necessary to use cells in the logarithmic phase of multiplication for certain experiments.

Nucleic Acid Synthesis in Cells Infected in the Lag Phase.—The best system

to analyze for nucleic acid synthesis during virus formation would be one in which the formation and release of phage take place over a fairly long period of time without cellular lysis. Such an experimental system is found when cells are infected in the lag phase (1). The results obtained under these conditions, however, are not strictly reproducible. Three distinct types of nucleic acid curves were obtained in 14 experiments. These will be designated group I, II, and III. Five are in one group, 6 in the second group, and 3 in the third group.

All experiments could be classified under one of the three groups. No experiments were intermediate between the groups.

The experiments that fall in the first group all show an increase in cell RNA after infection, with a continual gain in total RNA and DNA during the course of phage formation. In the second group, the cells show a loss of cell RNA after infection, although there is a continual gain of total RNA and DNA in the infected suspension. The third group consists of experiments which show a total loss of RNA from the suspension at about the time when most of the virus appears in the medium.

In all three groups there was a release of RNA and DNA into the medium. It should be mentioned here that although the results have been expressed in terms of free ribonucleic acid released into the medium, it is possible that ribonucleoprotein not free ribonucleic acid is released from the infected cells into the medium, since a large residue remains after heating the 2.5 per cent trichloroacetic acid suspension from the medium in 5 per cent trichloroacetic acid for 15 minutes at 90°C.

Group I

The results of the experiments in the first group are plotted in in Fig. 2 A, in which values are for the total suspensions. The points are the averages of 5 separate experiments. Three times as many phage particles as cells are added to the infected suspension. All cells are infected after 20 minutes, as shown by the fact that the cell count falls to zero in the infected suspension. For the first 40 to 50 minutes there is little, if any, change in any of the determined components. At the end of this time, there is an increase in the total nucleic acid of both infected and uninfected suspensions, the increase being a little more in the infected suspension. At 100 minutes there is more DNA synthesized in the infected suspension than in the uninfected suspension while the amount of RNA synthesized is about the same in either suspension; hence, the amount of total nucleic acid in the infected suspension is somewhat higher than in the uninfected suspension. The bacterial count in the normal suspension has increased from 3.0×10^{10} cells to 4.75×10^{10} cells, while the infected bacterial count has remained approximately constant, with the phage titer going from 0.9×10^{11} particles to 6.0×10^{11} particles.

In Fig. 2 B the results are expressed in terms of nucleic acid per 3.0×10^{10}
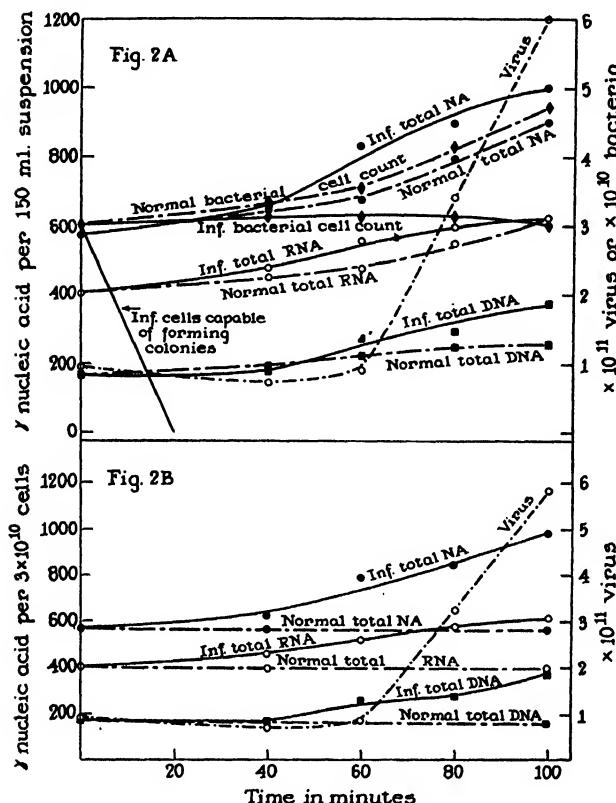
FIG. 2 A. Nucleic acid, bacteria, and virus changes of normal and infected lag phase cells for 150 ml. of suspension.

Cells were washed off from a 22 hour veal infusion slant and prepared as described under Methods. Erlenmeyer flasks, containing synthetic medium, were inoculated with the bacteria to give 2.0×10^8 b/ml. and shaken at 37°C. for 1 hour. At this time phage was added to the infected samples, as described under Methods. Both control and infected sample were then pipetted into sterile 2.0×15 cm. test tubes as in Methods and the tubes shaken at 37°C. At the indicated times, 15 control tubes and 15 infected tubes were treated, as described under Methods. Bacteria, virus, and nucleic acid were determined as in Methods. The curve represents the average plot of 5 separate experiments.

FIG. 2 B. Nucleic acid of normal and infected lag phase cells for 3.0×10^{10} cells. Experimental conditions as described in Fig. 2 A.

cells. On this basis the infected system has a higher content of RNA, DNA, and NA than the normal system. This is due to the fact that whereas the infected cells cannot multiply, the normal bacteria do, and thus while the nucleic acid content of the suspension is approximately the same in both instances, the infected system has a higher nucleic acid content when calculated per cell.

Although the total nucleic acid content of the infected suspension is only slightly greater than that of the uninfected suspensions as shown in Fig. 2 A, the the distribution of the nucleic acid is entirely different in the two cases. From
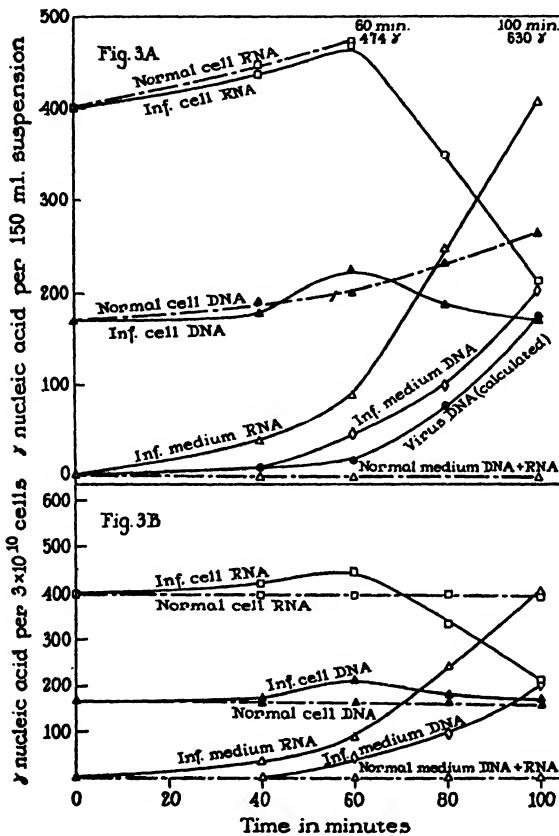
FIG. 3 A. Nucleic acid content of normal and lag infected bacteria per 150.0 ml. of suspensions.

Experimental conditions as described in Fig. 2 A.

FIG. 3 B. Distribution of nucleic acid of normal and lag infected bacteria per 3×10^{10} cells.

Experimental conditions as described in Fig. 2 A.

Fig. 3 A, which is expressed as gamma NA per 150 ml., it can be seen that infected cells release RNA and DNA into the medium, whereas normal cells release neither RNA nor DNA into the medium. Furthermore, practically all the DNA released into the medium from the infected cells is virus DNA.

Fig. 3 B shows the distribution of the various nucleic acids calculated per 3.0×10^{10} cells. The content of RNA and DNA per 3.0×10^{10} cells is higher in

the infected cells than the uninfected cells, due to the smaller number of bacteria present in the infected sample during the course of the infection.

The results, as a whole, show that normal bacterial suspensions and infected suspensions both synthesize approximately the same total quantity of nucleic acid. The infected cells synthesize about 30 per cent more DNA than normal cells (Fig. 2 A), and this excess DNA is practically all phage (*cf.* Fig. 3 A). In the normal suspension the nucleic acid synthesized remains in the cells, which increase in number, while in the infected suspension the nucleic acid is liberated from the cells into the medium.

Group·II

The results obtained in the second group of experiments are shown in Fig. 4 A, where the content of nucleic acid of the total suspensions are plotted. The points are the average plots of 6 experiments. In this type of curve there is also approximately the same amount of nucleic acid synthesized in the normal and infected suspensions. There is more DNA synthesized and a little less RNA formed in the infected suspension. Group II differs from group I in that, as shown in Fig. 4 B, there is a loss of cell RNA after infection, and the increase in cell RNA, shown in Fig. 3 A, does not occur. After the initial loss of cell RNA, the cell RNA concentration may then remain the same or increase slightly until virus begins to be liberated into the medium, at which time there is a further loss of RNA from the cell. Here again, most of the DNA appearing in the medium appears to be virus DNA.

Group III

The third type of nucleic acid curve is shown in Fig. 5 A. The points are the average plots of three experiments. All values are expressed in terms of gamma NA for 150 ml. of suspension. In this type of curve there is a loss of RNA from the total system, a gain in DNA, with the total nucleic acid concentration remaining the same during the interval between 80 to 110 minutes (*cf.* Fig. 5 A). In the interval between 80 and 110 minutes there is a decrease of 115 γ of RNA in the system. At the same time there is a gain of 127 γ of DNA. This gain in DNA is very nearly equal to the amount of DNA found in the virus; the result suggests the possibility of the conversion of RNA to virus DNA under certain conditions. These changes are shown in detail in Fig. 5 B, where the nucleic acid content of the cells and medium are plotted. Between 80 and 110 minutes there is a loss of 230 γ RNA from the cell of which 115 γ is found in the medium. Thus, 115γ of RNA is lost from the total system. The cell DNA at this time fell from 180 γ to 112γ or a loss of 68 γ of DNA from the cells. However, the DNA in the medium from 80 to 110 minutes rose from 30 γ to 185 γ or an increase of 155 γ of DNA. There is thus a loss of 68 γ of DNA from the cell, but 185 γ DNA appears in the medium; *i.e.*, a total gain of 127 γ DNA in the whole
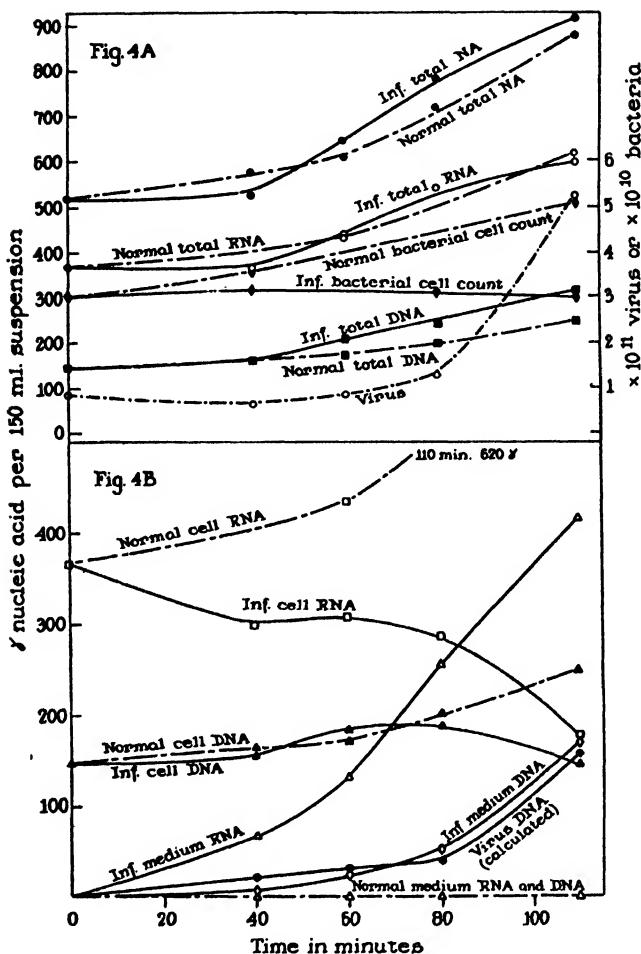
FIG. 4 A. Nucleic acid, bacteria, and virus changes of normal and infected lag phase cells for total suspension.

Experimental conditions as described in Fig. 2 A. The curves represent the average plot of 6 experiments.

FIG. 4 B. Distribution of nucleic acid of normal and infected lag phase bacteria for total suspension.

Experimental conditions as described in Fig. 2 A.

suspension. Adding the 115 γ of RNA lost to the 68 γ of DNA released from the cell gives a value of 183 γ of nucleic acid. This compares very favorably to the 185 γ of DNA actually found in the medium. It should be emphasized that much more evidence will have to be obtained to prove that a conversion of RNA to DNA takes place.
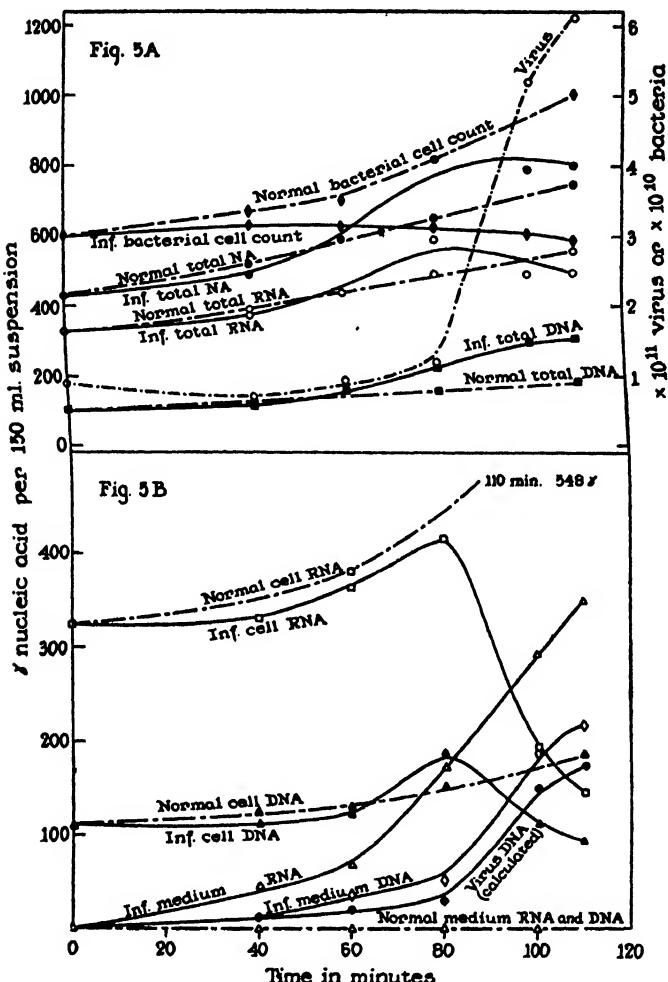
FIG. 5 A. Nucleic acid, bacteria, and virus changes of normal and lag phase infected bacteria for total suspension.

Experimental conditions as described in Fig. 2 A. The curves represent the average plot of 3 experiments.

FIG. 5 B. Distribution of nucleic acids of normal and infected lag phase bacteria for total suspension.

Experimental conditions as described in Fig. 2 A.

Relation of DNA in Medium to Virus.—Chemical analysis of the phage used in this work gives between 0.24 γ and 0.30 γ of DNA per 10^9 plaques. Table I shows a typical experiment. Apparently most of the DNA in the medium of lag phase infected cell suspensions is virus DNA. This is shown in Figs. 3 A, 4 B,

and 5 B, in which the DNA found in the medium is compared to the DNA calculated from the amount of virus appearing in the medium.

This relation between virus DNA and total DNA in the medium is confirmed by the results obtained when the solution is centrifuged (Table II). The decrease

TABLE I

Chemical Analysis of Centrifuged Phage

300 ml. of phage prepared in synthetic medium, having a titer of 1.2 × 10¹⁰ plaques per ml. and prepared as under Methods, was centrifuged for 8 minutes at 3600 R.P.M. (1200 × *g*) at 10°C. The supernatant fluids were poured off and recentrifuged 2 hours at 12,000 R.P.M. (14,000 × *g*) at 10°C. The supernatant fluid was poured off, and the virus precipitates combined in 25.0 ml. of synthetic medium. This solution was centrifuged for 5 minutes at 3600 R.P.M. (1200 × *g*) at 10°C. The supernatant fluid was recentrifuged 90 minutes at 12,000 R.P.M. (14,000 × *g*) at 10°C. The virus precipitate was made up to 5.0 ml. with synthetic medium and assayed for virus titer. The remaining solution was chilled in ice, and 10 per cent trichloroacetic acid added to give a final concentration of 2.5 per cent trichloroacetic acid and left 1 hour at 5°C. The precipitate was centrifuged at 3600 R.P.M. (1200 × *g*) at 10°C., washed once with 5.0 ml. of 2.5 per cent cold trichloroacetic acid, and suspended in 2.5 ml. of 5 per cent trichloroacetic acid. Nucleic acid was then determined as in Methods.

Total phage count	γ total DNA	γ total RNA	$\dfrac{\gamma \text{ DNA}}{10^9 \text{ phage}}$	$\dfrac{\gamma \text{ RNA}}{10^9 \text{ phage}}$
611 × 10⁹	161	20.3	0.28	0.033

TABLE II

Relation of DNA in Medium to Virus

The total DNA and virus titer in the medium was determined on 150 ml. at 90 minutes in an infected sample from an experiment, as shown in Fig. 2 A. Another 150 ml. sample identical with the first sample was centrifuged 60 minutes at 12,000 R.P.M. (14,000 × *g*), and the total DNA and virus titer determined in the supernatant fluid. The figures below show the virus titer and DNA content before and after centrifuging.

Sample	Total phage count	γ total DNA	$\dfrac{\gamma \text{ DNA}}{10^9 \text{ phage}}$
Before centrifuging..........................	640 × 10⁹	189	0.30
After centrifuging............................	420 × 10⁹	139	0.33

in DNA is just proportional to the decrease in phage content of the supernatant fluid.

Nucleic Acid Synthesis of Log Phase Infected Cells.—In order to determine whether the total nucleic synthesis of infected suspensions is the same as normal suspensions which are synthesizing nucleic acid in their greatest quantities, it is better to use cells in the log phase. At this time the normal bacteria have their greatest concentration of nucleic acid, and the amount per cell remains
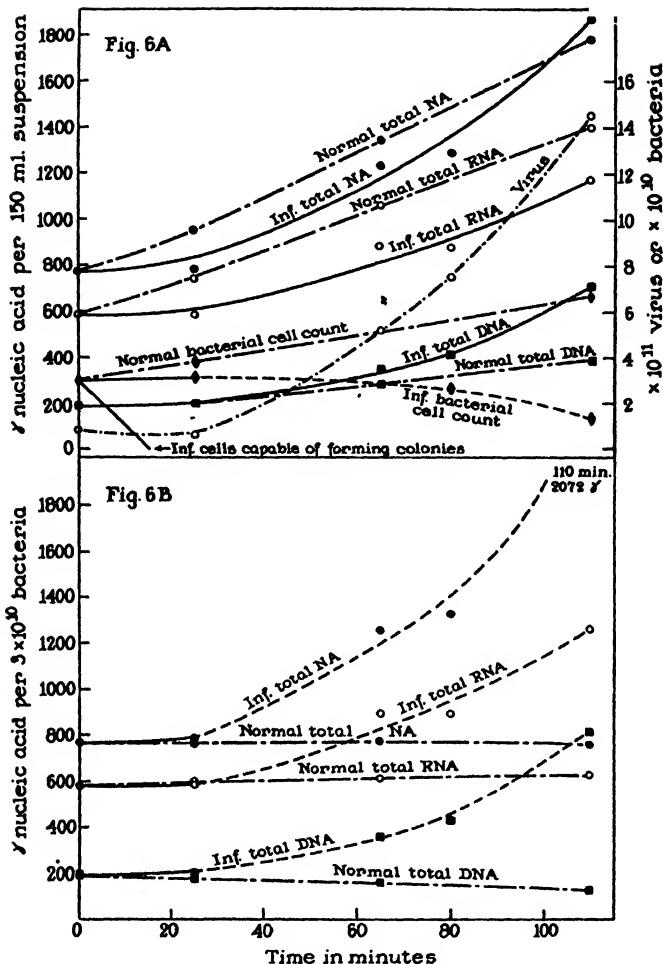
FIG. 6 A. Nucleic acid, bacteria, and virus changes of normal and log phase infected bacteria for total suspension.

Cells were washed off a 22 hour veal infusion slant and prepared as described under Methods. Erlenmeyer flasks, containing synthetic medium, were inoculated with 8.0×10^7 washed cells and shaken for $3\frac{1}{2}$ hours at 37°C. At this time the cell count was about 2.0×10^8 cells per ml. Phage was added to the infected sample, as described under Methods, and samples were then pipetted into 2.0×15 cm. test tubes and shaken at 37°C. as in Methods. At the indicated times, 15 control tubes and 15 infected tubes were removed and treated as described in Methods. Bacteria multiplication, virus assay, and nucleic acid were determined as in Methods. The curves represent the average plot of 5 experiments. Cellular lysis started between 60 to 65 minutes.

FIG. 6 B. Nucleic acid of normal and log phase infected bacteria for 3.0×10^{10} cells.

Experimental conditions as described in Fig. 6 A.

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fairly constant during the period of virus formation and liberation. This sytem has the disadvantage that virus liberation occurs a fairly short time after infection and the cells undergo lysis. The cell count of the normal suspension also increases fairly rapidly during this time.

The results show, however, that virus infected log phase bacterial suspensions synthesize approximately the same amount of nucleic acid as normal log phase bacterial suspensions.

Five experiments were carried out with log phase cells. The results were averaged and plotted in Figs. 6 and 7.

Fig. 6 A shows the plot of 5 experiments expressed as gamma nucleic acid per 150 ml. of suspension. The total nucleic acid is approximately the same in nor-

TABLE III

Changes in Relative Nucleic Acid Ratios in Normal and Infected Suspensions

Data derived from Fig. 6 A.

				Infected suspension		
Sample time	DNA	RNA	$\frac{RNA}{DNA}$	$\frac{DNA\ time}{DNA\ initial}$	$\frac{RNA\ time}{RNA\ initial}$	Total phage $\times 10^{11}$
min.	γ	γ				
0	190	580	3.06	1.00	1.00	0.9
25	200	585	2.93	1.05	1.02	0.35
65	350	880	2.52	1.84	2.32	6.1
80	415	870	2.10	2.28	2.29	7.5
110	720	1170	1.63	3.79	3.08	14.5
				Normal suspension		
0	190	580	3.06	1.00	1.00	
25	200	745	3.73	1.05	1.96	
110	385	1405	3.66	2.02	3.70	

mal and infected suspensions. The normal suspensions show a greater synthesis of RNA, while the infected suspension has a greater DNA content. Table III shows the ratios of RNA and DNA synthesized in normal and infected suspensions at various times.

In Fig. 6 B the various nucleic acids are expressed as gamma NA per 3.0×10^{10} cells. Calculated in this manner, the infected system shows a much higher content of RNA, DNA, and NA than the normal system.

Although the total nucleic acid is the same in normal and infected log phase bacterial suspensions, the distribution is different. Fig. 7 A shows that there is a continual loss of cell RNA shortly after infection. There is also a loss of cell DNA. There appears to be more DNA synthesized than is necessary for the amount of virus formed. Normal suspensions release neither RNA nor DNA into the medium.

Fig. 7 B shows the distribution of the nucleic acid calculated per 3.0×10^{10} cells. On this basis the infected system has a higher content of nucleic acid, due to the fewer number of cells present.
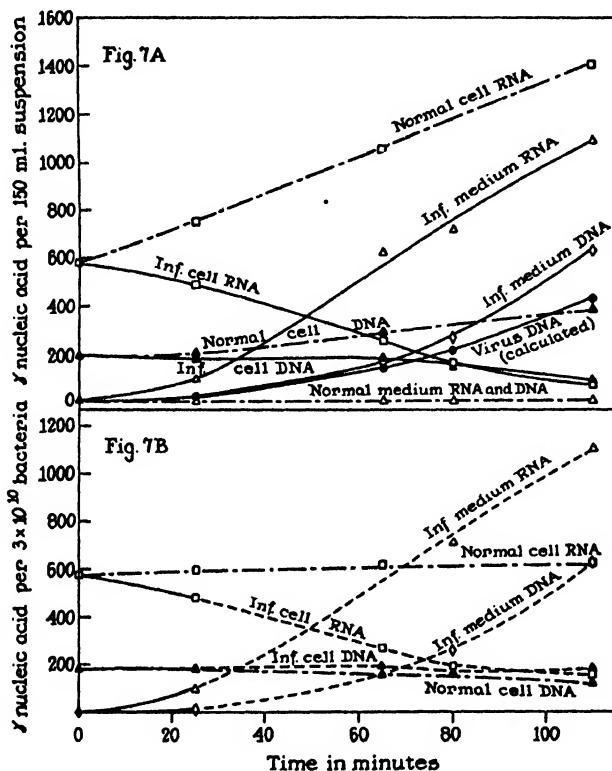
FIG. 7 A. Nucleic acid content of normal and log phase infected bacteria per 150.0 ml. of suspension.

Experimental conditions as described in Fig. 6 A. The dotted lines indicate the start of lysis. The correction for RNA and DNA per cell from this time on is open to error, since it is impossible to know the correct number of cells during lysis. The nucleic acid in the infected medium was not corrected per cell, and these values represent the medium of the total suspension. Cellular lysis started between 60 to 65 minutes.

FIG. 7 B. Distribution of nucleic acid of normal and infected log phase bacteria per 3.0×10^{10} cells.

Experimental conditions are described in Fig. 7. Dotted lines as in Fig. 6 A.

Another point brought out by these experiments is that all the virus cannot be present in the cell at the time liberation of the virus starts. This is shown in Fig. 6 A. To account for the total of 1.45×10^{12} virus particles formed in 110

minutes, it would be necessary for the infected system to contain 435 γ of DNA at 60 minutes when the cells begin to lyse. Actually, there is only 350 γ of DNA present at this time, of which 160 γ was synthesized after infection.

During the time that 50 per cent of the total virus is formed, that is, from 0.7×10^{12} particles to 1.45×10^{12} particles, there is a slow cellular disintegration and a slow increase in phage (Fig. 6 A). Most of the increase of virus DNA, however, is not observed in the cells (Fig. 7 A). One has to assume, therefore, that at this stage in the cycle, the virus is formed and released very rapidly from the cells, or else the synthesis does not take place in the cell under these conditions. This problem is now under investigation.
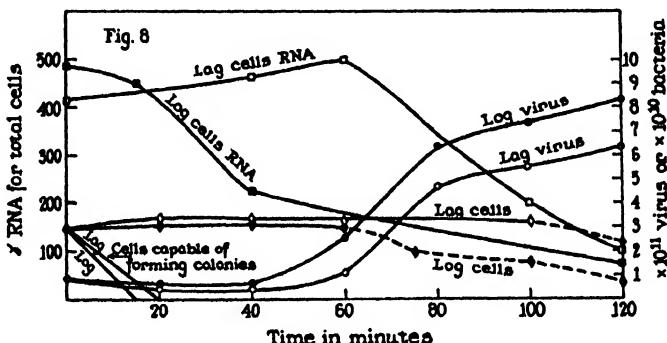
FIG. 8. Lysis of infected lag phase and log phase cells.

Lag phase and log phase infected cells were prepared as described in Fig. 2 A and 6 A respectively. At the indicated times, the suspensions were centrifuged and RNA determined on the cells as in Methods. Dotted lines indicate the start of cellular lysis.

Lysis of Infected Lag Phase and Log Phase Cells.—Fig. 8 shows that log phase infected cells lyse sooner than lag phase cells. Before lag phase infected cells lyse, there is also a continuous loss of cell RNA as in infected log phase cells. Both types of infected cells begin to lyse about 60 minutes after the cell RNA has decreased *continuously*. Lag phase cells sometimes lose cell RNA immediately after infection, as shown in Fig. 4 B. However, in contrast to infected log phase cells, they appear able to prevent this loss of cell RNA after a short time.

Whether this continuous loss of cell RNA is directly associated with the mechanism of cell lysis is not known. A point which indicates that such may be the case is the observation that this uninterrupted loss of cell RNA precedes any change in cell lysis (*cf.* Fig. 8). It would be predicted from this hypothesis that the yeast fraction, which accelerates the lysis of lag phase infected cells (4), would cause a steady loss of cell RNA in a shorter time than is usually observed.

DISCUSSION

Infected *S. muscae* bacterial suspensions in the log phase show about the same total amount of nucleic acid synthesis as normal cell suspensions when calculated from the beginning of infection to the completion of virus formation. Infected suspensions, however, synthesize very little, if any, nucleic acid for 35 to 40 minutes after infection in contrast to normal suspensions analyzed for the same time period (*cf.* Fig. 6 A). After this time, the infected suspensions synthesize nucleic acid a little faster than normal suspensions, so that at the end of virus formation there is approximately the same amount of nucleic acid present in infected and uninfected suspensions.[2]

Normal cell log phase suspensions have a RNA/DNA ratio of 3 to 4 while infected suspensions show an RNA/DNA ratio of 1.6 at the end of phage formation (*cf.* Table III). This results from the infected suspension synthesizing less RNA and more DNA than the control suspension.

Infected *S. muscae* cells in the lag phase synthesize both RNA and DNA. They release RNA into the medium well before cellular lysis begins and before any virus is released into the medium. Such cells release little or no DNA into the medium before virus liberation. Whether such cells show an increased RNA content over that present when the virus is added seems to depend upon a balance between RNA synthesis and RNA release. The mechanism which determines the release of RNA from the infected cells is not known. Normal cells release neither RNA nor DNA into the medium.

At the time the virus appears in the medium, there is a loss of cellular RNA below that initially present at the time of infection.[3] There is also a release of DNA from the infected cell at this time. The amount of DNA released seems to be the amount which was synthesized after infection, and the DNA content returns to the value found at the time the virus was added. The increased DNA content in these cells may, therefore, actually be the DNA in the virus particles.

As a working hypothesis, it is proposed that, with this particular strain of *S. muscae*, phage synthesis is closely related to normal cellular reactions, and not an entirely independent series of reactions (21). On virus infection, however, these reactions are shifted in such a manner as to result in the formation of phage, as shown by the increased DNA content of the infected suspension.

It is further suggested that the enzymes of the host are used for the synthesis of the genetic units of the virus with the virus determining how these "units" are put together to form the intact virus particle. This scheme would be the same whether the phage particle breaks up after infecting a cell and then reaggregation occurs, as suggested by Luria (7), or whether it remains intact.

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[2] In some experiments just completed, nucleic acid synthesis of infected suspensions was about 85 per cent of that found in normal suspensions.

[3] These cells still stain Gram-positive at this time. There is no evidence, therefore, that the RNA released from the cells is concerned with the Gram stain.

If phage synthesis does occur by Luria's mechanism, such a synthetic system may not be peculiar to phage, since there is some evidence that normal protein synthesis may occur by successive aggregation of definite units (8).

The above point of view explains some earlier results obtained with this system. Under normal conditions the rate of formation of phage is proportional to the rate of multiplication of normal cells under the same conditions (9). This is unexpected if the virus has a more or less independent synthesis. If, however, the virus synthesis is simply a modification of the synthesis occurring in normal cells, it would be expected that the two reactions would in general be parallel. The fact that under certain conditions (10, 11) the production of this phage may occur without multiplication of bacterial cells shows that at some point the synthetic reaction(s) governing bacterial multiplication and virus synthesis is different.

The fact that the normal cell system and the phage system compete for certain nutrients (3, 12) and that inhibition of the energy system for normal cellular synthesis also prevents phage formation (6) is also to be expected from the above point of view, since the basic synthetic reactions would be the same.

It is interesting to compare the effect of this virus on the nucleic acid metabolism of *S. muscae* to the effect of other viruses on the nucleic acid systems of their hosts.

Gratia, Brachet, and Jeener (13) reported that the silkworm jaundice virus caused an increase in the ribonucleic acid and desoxyribonucleic acid content of infected cells. The virus appears to have only desoxyribonucleic acid as its nucleic acid constituent. If it turns out that no ribonucleic acid is present in the *S. muscae* virus, then the effect of these two viruses on the nucleic acid metabolism of their hosts will be similar.

Cohen has reported that *E. coli* B infected with virus T2r+ synthesized only desoxyribonucleic acid, and that the ribonucleic acid content of the infected cell remains constant during the course of the infection (14). He has proposed that the desoxyribonucleic acid synthesized in the infected cell is the virus. He has furthermore indicated that only virus material is synthesized in the infected cell. The *E. coli* system differs from the system described in this paper in that the *S. muscae* system synthesizes both ribonucleic acid and desoxyribonucleic acid. The amount of ribonucleic acid present in the *S. muscae* purified virus is very small and may not be a consittuent of the virus, so that in this system it may turn out that the infected cell can synthesize more than virus material. All that can be said with certainty now is that the infected cell synthesizes far more ribonucleic acid than is needed for the formation of the virus.

Methods

Preparation of Bacteria.—Bacteria were grown for 22 hours on veal infusion agar at 36°C. The cells were then washed off with synthetic medium (1) and centrifuged at 3600 R.P.M. (1200 × g) for 10 minutes. The supernatant fluid was poured off, the

cells resuspended in 10 ml. of synthetic medium, and again centrifuged as before. Such washed cells were used for all experiments.

Cells in the lag phase and cells in the log phase were used in the various experiments. The lag phase cells were prepared by inoculating synthetic media with the washed cells described above to give 2.0×10^8 cells per ml. The cells were shaken for 1 hour at 36°C. before addition of the virus. Log phase cells were prepared by inoculating synthetic medium with 8.0×10^7 cells per ml. and shaking the mixture until the cell count was about 2.0×10^8 cells per ml. This took about 3½ hours.

Bacteria were determined by the turbidimetric method described previously (10), unless otherwise stated.

The stock culture was transferred every 24 hours on veal infusion agar slants and grown at 36°C. The culture was a mucoid one and gave practically all yellow colonies when plated on tryptose phosphate agar. In the synthetic medium used, it had a division time of about 2 hours and a lag period of between 60 and 75 minutes.

Preparation of Virus.—500 ml. of synthetic medium in a 1000 ml. Erlenmeyer flask was inoculated with washed cells to give 6.0×10^7 cells per ml. This mixture was shaken for 4 hours at 36°C. at the end of which time the cell count was 2.0×10^8 cells per ml. 30 ml. of synthetic phage prepared as described previously (1), which had a titer of approximately 1.0×10^{10} particles per ml., was added to the 500 ml. of cells. This mixture was shaken, and then 10 ml. samples were pipetted into 2.0×15 cm. test tubes. These tubes were then shaken for about 2 hours at 36°C. The contents of the tubes were centrifuged for 8 minutes at 3600 R.P.M. ($1200 \times g$) at 5°C. The supernatant fluids were combined and recentrifuged for 2 hours at 12,000 R.P.M. ($14,000 \times g$) at 10°C. The supernatant fluids were poured off, the tubes allowed to drain on filter paper, and the precipitates containing the virus combined in a total of 7 ml. of synthetic medium and stored at 5°C. Virus was assayed as previously (15). All dilutions were carried out in 1 per cent veal infusion medium, using a new pipette for each dilution.

Medium.—The same synthetic medium was used as described previously (1). 5 mg. of acid-hydrolyzed casein and 0.05 ml. of 0.1 M calcium chloride was added to every 10 ml. of medium.

Example of an Experiment for Determining Changes in Nucleic Acid Content of Bacterial Suspensions[4].—The procedure to be described was found to give the most reproducible results, the number of flasks used depending on the amount of reaction mixture required. The following procedure was carried out with cells in the log phase. Experiments with cells in the lag phase were carried out in the same way, except that the medium was inoculated with 2.0×10^8 cells per ml. and shaken only 1 hour. All reaction mixtures were finally shaken in 2.0×15 cm. test tubes.

Time 9:30 a.m. Eight 500 ml. Erlenmeyer flasks were set up containing 200 ml. of synthetic medium in each one. Bacteria from a 22 hour old culture grown on veal infusion agar were prepared as described above. Each of the 6 flasks was inoculated with 8.0×10^7 cells per ml.

1:00 p.m. All flasks were shaken for 3½ hours and poured into one big flask and well mixed. The flask was kept at 37°C. The cell count had risen to 2.0×10^8 cells per ml. From this mixture 10.0 ml. samples were pipetted into each of 45 tubes kept

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[4] In all experiments described in this paper, there was approximately 85 per cent adsorption of the virus.

at 37°C. The remaining solution was inoculated with 1.0 ml. of virus for every 10.0 ml. of solution. Enough virus was added so that all the cells were infected; in this case, 7.1 × 10⁸ virus particles per ml. 11.0 ml. samples of the bacterial-virus mixture were then pipetted into 90 tubes kept at 37°C. The 135 tubes were shaken at 37°C.

1:15 p.m. The 15 control tubes and 15 infected tubes were removed from shaker and turbidity measurement taken for cell count. Viable cell counts were made by removing 2.0 ml. samples from 2 infected tubes and 2 control tubes, centrifuging them, washing cells once with veal infusion and suspending the two infected samples in 2.0 ml. of veal each, then pipetting into sterile Petri dishes and adding 8.0 ml. of veal infusion agar. Control cells were first diluted five times using a 1/10 dilution each time and were then pipetted into Petri dishes and veal infusion agar added.

1:16 p.m. 15 control tubes and 15 infected tubes were plunged into ice-salt mixture.

1:18 p.m. The contents of both tubes were poured into cooled lusteroid centrifuge tubes and centrifuged for 5 minutes at 10°C. at 3600 R.P.M. (1200 × g). Each tube held 25 ml.

1:26 p.m. The supernatant fluids were poured off. The control supernatants were poured into a 200 ml. Erlenmeyer flask. Both flasks were in an ice bath. A virus assay was made on the infected supernatant fluid by making two 7 × 1/10 dilutions and plating 1.0 ml. of each final dilution onto two agar plates. 1.0 ml. was removed from the control supernatant to compensate for the 1.0 ml. taken from the infected supernatant fluid for virus assay. To both flasks was then added 8.0 ml. of cold 50 per cent trichloroacetic acid. The flasks were then put at 5°C. until the following day.

1:29 p.m. All the control cells were combined, and all the infected cells were combined. Both samples were then suspended in 25.0 ml. of ice cold saline.

1:33 p.m. Cells centrifuged.

1:40 p.m. Cells suspended in 5.0 ml. of 10 per cent cold trichloroacetic acid. At the required times all samples were treated as described above.

Nucleic Acid Determinations

Preparation of Cells.—The cells which had been suspended in 5.0 ml. of ice cold 10 per cent TCA were poured off into a chilled mortar. The centrifuge tube was rinsed with 3.0 ml. of ice cold 10 per cent TCA, and this was also poured into the mortar. A pinch of alundum was added to the mortar, and the cells were ground mechanically (Eimer and Amend mechanical mortar) for 20 minutes. The ground cells were poured into a centrifuge tube and the mortar rinsed with 3.0 ml. of ice cold 10 per cent TCA. This sample was centrifuged for 10 minutes at 3600 R.P.M. (1200 × g) at 5°C. The supernatant fluid was poured off and the cells suspended in 5.0 ml. of ice cold 6 per cent TCA and poured back into the ice cold mortar. The centrifuge tube was rinsed with 3.0 ml. of 6 per cent TCA which was added to the chilled mortar. The cells were ground another 20 minutes. The contents were then poured out into a centrifuge tube, the mortar rinsed with 3.0 ml. of ice cold 6 per cent TCA, which was added to the centrifuge tube, and the sample centrifuged as described above. The supernatant fluid was poured off and the tube allowed to drain on a piece of filter paper. The residue was then washed with 10.0 ml. of cold 6 per cent TCA and centrifuged as above. These two extractions were sufficient to remove all the acid-soluble substances which would interfere with the determination of nucleic acid (Table IV). The cells were then suspended in 1.5 ml. of 5 per cent TCA and heated 15 minutes at 90°C. (16).

The sample was cooled for a few minutes, and centrifuged, and the supernatant poured off. The residue was reextracted in 1.5 ml. of 5 per cent TCA at 90°C. for 15 minutes, cooled, centrifuged, and the supernatant added to the original supernatant fluid. This solution was again centrifuged for 5 minutes at 3600 R.P.M. (1200 × g). The supernatant was then poured off and kept for the nucleic acid determination.

Analysis of Medium.—The supernatant fluid from the centrifuged suspension cell was adjusted to 2.5 per cent TCA and kept overnight at 5°C. The suspensions were then centrifuged for 10 minutes at 3600 R.P.M. (1200 × g) at 5°C. The precipitates were washed once with 10.0 ml. of ice cold 5 per cent TCA and again centrifuged as above. The precipitate was suspended in 1.5 ml. of 5 per cent TCA and heated 15 minutes at 90°C., cooled, and centrifuged. The supernatant was poured off, and the residue reextracted as above. The two filtrates were combined. This filtrate represented all the nucleic acid and protein in the medium.

Reliability of Method.—Several duplicate samples run through this procedure agreed within ± 5 per cent. As a further check on the method, known amounts of yeast nucleic acid and thymonucleic acid were added to infected cells in the lag phase and infected cells in the log phase. Table V shows the recovery was 90 per cent or over for both desoxyribonucleic acid and ribonucleic acid.

Accuracy of Calculating the Average Error of the Mean for Nucleic Acid Determinations

The average error of the mean for the various nucleic acid determinations of different experiments for the same time interval was calculated from the formula

$$\frac{\Sigma(+v)}{n\sqrt{n}}$$

where $\Sigma(+v)$ equals the sum of the differences from the mean and η equals the number of experiments (19).

The average error of the mean for lag phase infected cells was from 5 per cent to 15 per cent in groups 1, 2, and 3. For log phase infected cells, it was from 5 per cent to 20 per cent.

Preparation of Standard Curves—Color Tests.—Purified yeast nucleic acid and thymus nucleic acid[5] were used to prepare the standard curves for the orcinol test (17) and the diphenylamine test (18). Since the experimental samples were heated in 5 per cent TCA in order to extract the nucleic acid, the standard samples were also heated in 5 per cent TCA before being put through the color test. The samples were read in a Beckman spectrophotometer at wave lengths of 660 mμ and 600 mμ in quartz cells. Figs. 9 and 10 show the standard orcinol and desoxyribonucleic curve used in these experiments. Since ribonucleic acid only showed a 1 per cent color value with the desoxyribonucleic reagent, no correction was made for this. However, as reported by

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[5] Purified yeast nucleic acid prepared by Dr. M. Kunitz and purified thymus nucleic acid prepared by Dr. A. Mirsky were used as standards. I wish to thank Dr. Kunitz for these samples.

TABLE IV

Extraction of Acid-Soluble Substances Showing a Maximum at 260 mμ

Infected lag phase and log phase cells were prepared as described in Figs. 2 A and 6 A, respectively, and extracted as in Methods. The first was a 10 per cent trichloroacetic acid (TCA) extract. The cells were reextracted with 6 per cent TCA as in Methods. The cells were then reextracted a third time in 3.0 ml. of 6 per cent TCA. As the trichloroacetic acid extraction removes nucleotides, nucleosides, and all acid-soluble components of nucleic acid, it is very difficult to know the exact amount of such materials that one is extracting. The results are, therefore, expressed as the amount of material showing a maximum at 260mμ, which is extracted with each extraction compared to total amounts of such acid-soluble material. The third extract gave no reaction when tested with the orcinol reagent (17) or the diphenylamine reagent (18).

The method was chosen instead of phosphorus determination of the various extracts because nucleosides and perhaps other acid-soluble building blocks, which would interfere with the determination of nucleic acid, do not contain phosphorus. Under these conditions the most sensitive test is the determination of acid-soluble substances, which show a maximum at 260 mμ.

Extract	Per cent of total extracted material showing a maximum at 260 mμ
First (10 per cent TCA).....................................	70–85
Second (6 per cent TCA)....................................	15–30
Third (6 per cent TCA).....................................	0

TABLE V

Recovery of Added Ribonucleic Acid (RNA) and Desoxyribonucleic Acid (DNA) from Log and Lag Phase Infected Cells

Cells were prepared as described in Fig. 2 A to obtain lag phase infected cells, and log phase infected cells were prepared as described in Fig. 6 A. At the indicated times, lag phase cells were centrifuged out and suspended in 10.0 ml. of cold saline as were the log phase cells. Both lag and log cells were then divided into 5.0 ml. portions and centrifuged. The cells not receiving added nucleic acid were suspended in 4.0 ml. of 10 per cent trichloroacetic acid (TCA). The cells getting the added nucleic acid were suspended in 2.0 ml. of 20 per cent TCA. To these samples was then added 2.0 ml. of water containing 700 γ RNA and 400 γ DNA. Nucleic acid was then determined as in Methods.

Time	Type of cells	Total cell RNA	Total cell RNA + mixture	Δ	Total cell DNA	Total cell DNA + mixture	Δ
min.		γ	γ	γ	γ	γ	γ
20	Lag phase	357	1009	652	141	509	348
40	" "	251	911	661	158	513	375
65	" "	408	1061	653	198	569	371
90	" "	254	924	670	132	521	389
20	Log phase	442	1111	669	153	521	368
50	" "	302	976	674	123	499	376
85	" "	103	824	721	96˙	481	385
Average found, γ...........................				671			373
Amount added, γ...........................				700			400
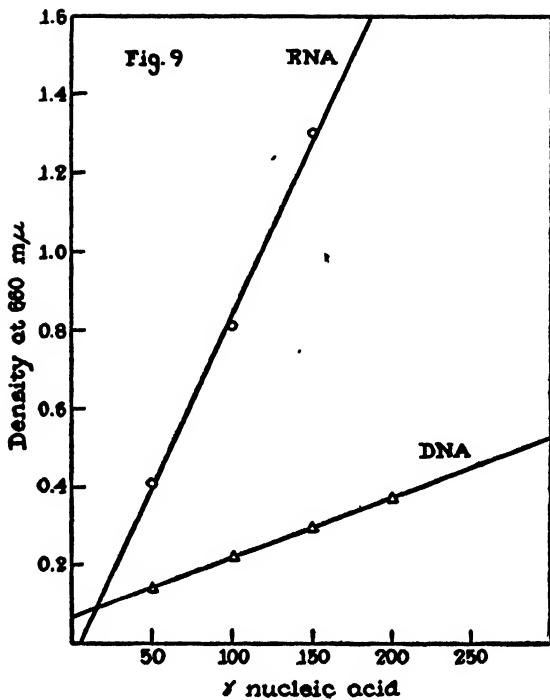
FIG. 9. Standard curve for ribonucleic acid determined by the orcinol test. This figure also shows that desoxyribonucleic acid reacts with the orcinol reagent. Curve determined as in Methods.
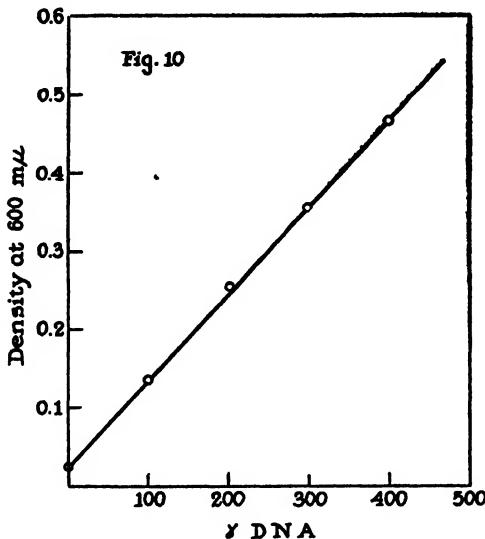
FIG. 10. Standard curve for desoxyribonucleic acid determined by the diphenyl-amine test. Curve determined as in Methods.

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Schneider (16), desoxyribonucleic acid gives quite a high value with orcinol reagent. In our hands, the desoxyribonucleic acid gave a 10 per cent color value with the orcinol reagent (Fig. 9). Therefore, all ribonucleic values were corrected for this amount.

Ultraviolet Absorption Curve.—Fig. 11 shows a standard ultraviolet absorption curve of purified yeast nucleic acid and desoxyribonucleic acid. Two facts should be
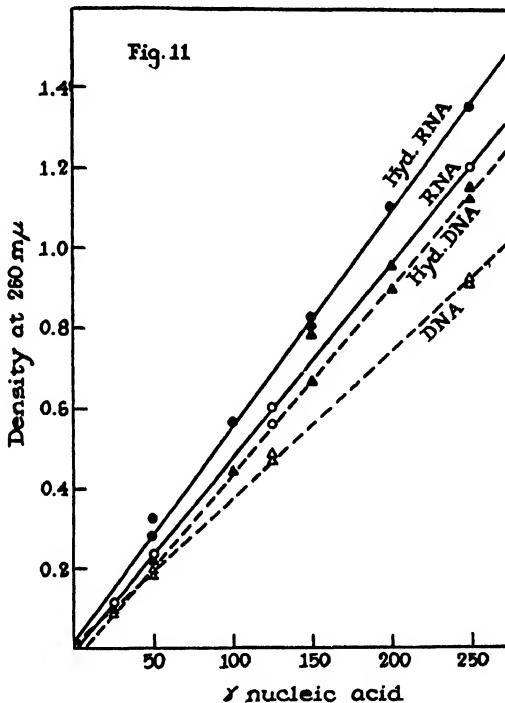
FIG. 11. Ultraviolet absorption curve of ribonucleic and desoxyribonucleic acids. This figure shows that hydrolyzing ribonucleic acid and desoxyribonucleic acid with 5 per cent trichloroacetic for 15 minutes at 90°C. increases the ultraviolet absorption of the nucleic acids. The values were corrected for the reading trichloroacetic acid gave alone. Curve determined as in Methods.

mentioned. One, since ribonucleic acid gives a slightly higher absorption than desoxyribonucleic acid, the experimental samples were read off the ribonucleic curve, since they contained more ribonucleic acid than desoxyribonucleic acid. Secondly, the standard solutions of ribonucleic acid and desoxyribonucleic acid heated for 15 minutes in 5 per cent TCA gave slightly higher values than the unheated samples. Since the experimental samples were hydrolyzed in 5 per cent TCA, the standard curve used in these studies was obtained by heating the known amount of desoxyribonucleic and ribonucleic acid under exactly the same conditions as the experimental samples were heated. All readings for the standard curves and experimental samples were read in

quartz cells at a wavelength of 260 mμ in a Beckman spectrophotometer. Total nucleic acid was calculated from the ultraviolet absorption spectrum of the sample based upon the maximum reading at a wave length of 260 mμ.

Phosphorus.—Phosphorus was determined by the method of King (20). The amount of nucleic acid calculated from the phosphorus determination, the color tests, and the ultraviolet absorption measurement agreed to within ± 15 per cent. Fig. 12 shows the standard curves for the phosphorus determination of ribonucleic and desoxyribonucleic acid..The solutions were all read at a wave length of 810 mμ in the Beckman spectrophotometer in quartz cells.
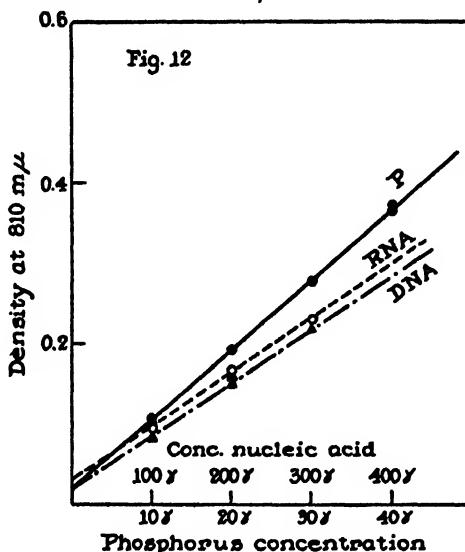
FIG. 12. Phosphorus standard curve. This figure shows the amounts of phosphorus present in the standard preparation of ribonucleic and desoxyribonucleic acids. Curve determined as in Methods..

In running each determination for nucleic acid, at least two different concentrations of standard solutions of both desoxyribonucleic acid and ribonucleic acid were run in duplicate.

I wish to thank Dr. John H. Northrop for his criticism during these experiments and for his help in the preparation of the experimental results for publication.

SUMMARY

1. The total nucleic acid synthesized by normal and by infected *S. muscae* suspensions is approximately the same. This is true for either lag phase cells or log phase cells.

2. The amount of nucleic acid synthesized per cell in normal cultures increases during the lag period and remains fairly constant during log growth.

3. The amount of nucleic acid synthesized per cell by infected cells increases during the whole course of the infection.

4. Infected cells synthesize less RNA and more DNA than normal cells. The ratio of RNA/DNA is larger in lag phase cells than in log phase cells.

5. Normal cells release neither ribonucleic acid nor desoxyribonucleic acid into the medium.

6. Infected cells release both ribonucleic acid and desoxyribonucleic acid into the medium. The time and extent of release depend upon the physiological state of the cells.

7. Infected lag phase cells may or may not show an increased RNA content. They release RNA, but not DNA, into the medium well before observable cellular lysis and before any virus is liberated. At virus liberation, the cell RNA content falls to a value below that initially present, while DNA, which increased during infection falls to approximately the original value.

8. Infected log cells show a continuous loss of cell RNA and a loss of DNA a short time after infection. At the time of virus liberation the cell RNA value is well below that initially present and the cells begin to lyse.

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A COMPARISON OF DESOXYRIBONUCLEIC ACID CONTENT IN CERTAIN NUCLEI OF NORMAL LIVER AND LIVER TUMORS

By DONALD D. MARK* and HANS RIS

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Direct chemical analyses of actively growing tissues, particularly tumors, for desoxyribonucleic acid have been reported by several investigators with, however, scant agreement. The percentage of desoxyribonucleic acid in nuclear material was found by Dounce[1] to be the same for Walker carcinosarcoma 256 and normal liver; in hepatoma 31 the percentage of desoxyribonucleic acid was lower than the normal. Brues, Tracy and Cohn[2] observed that the phosphorus and nitrogen content of hepatoma 31 and normal liver was the same per unit weight. On a similar basis, Davidson and Waymouth[3] and Schneider[4] recorded values for desoxyribonucleic acid that were higher in hepatomas than in normal liver.

Using the light absorption of fields of Feulgen stained nuclei, Stowell[5] also found higher values for the desoxyribonucleic acid in various tumors; here the results represented the average amount of desoxyribonucleic acid in many nuclei. The actual amount of desoxyribonucleic acid per single nucleus was not determined, nor was there any assay of the partition of the desoxyribonucleic acid into nuclear classes bearing different numbers of chromosomes.

Recent chemical determinations of the desoxyribonucleic acid in isolated nuclei by Boivin et al.,[6] Vendrely and Vendrely,[7] and Mirsky and Ris,[8] have demonstrated a remarkable species constancy in the desoxyribonucleic acid content of nuclei with the same number of chromosomes. In view of these findings, an attempt was made to establish the validity of this relationship for the presumably abnormal nuclei of tumors.

In the present study the amount of desoxyribonucleic acid in single nuclei of the same size has been compared in normal and tumor tissue utilizing the in-

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tensity of the Feulgen reaction. Precise data on the desoxyribonucleic acid content of single nuclei obtained by Mirsky and Ris[8] made it possible to use the Feulgen reaction as a measure of the relative amount of desoxyribonucleic acid per nucleus (Ris and Mirsky[9]).

The normal and tumor tissues studied were taken from the white rat. Liver tumors, hepatomas and cholangiomas, were induced by a diet of brown rice and carrot containing 0.06% p-dimethylaminoazobenzene as originally reported by Kinosita,[10] and were histologically identified according to the criteria described by Opie.[11] Tissue blocks were fixed in 10% formalin and cut at 10 μ thickness.

As elaborated elsewhere (Pollister and Ris;[12] Ris and Mirsky,[9]) the apparatus used in the measurements consisted of a Spencer monocular microscope with a mercury vapor arc as light source and a phototube and galvanometer stationed above a variable diaphragm in the image plane. The light absorption of in-

TABLE I

Relative Amount of Desoxyribonucleic Acid in the Nuclei of Normal Liver and of Liver Tumors

Cell type	No. nuclei	(E) extinction coefficient	(A) nuclear area	(E) × (A) relative amount of DNA
		Slide 1		
Normal hepatic...............	10	.161–.187 (.177)	23	4.09 ± .14
Hepatoma	10	.237–.260 (.247)	16.5	4.09 ± .12
		Slide 2		
Normal hepatic...	5	.208–.244 (.227)	23	5.22 ± .20
Cholangioma	10	.222–.265 (.225)	23	5.19 ± .23

dividual nuclei stained by the Feulgen reaction was measured at a wave length of 546 mμ

In Table I comparative measurements are shown of the smallest spherical nuclei in Feulgen stained paraffin sections of normal and tumor tissue mounted together on the same slide. The relative value for desoxyribonucleic acid per nucleus in the normal cell and in the hepatoma cell appears the same, as in slide 1. Similarly, slide 2 indicates the same desoxyribonucleic acid content per nucleus for the normal hepatic cell and the cholangioma cell.

Since the method is limited to the measurement of spherical nuclei, it was not possible to compare irregular bile duct nuclei with cholangioma nuclei directly, or to measure large nuclei of bizarre shape. Certain variations may be found in the values if the Feulgen technic varies slightly, as in Table I. Only sections on the same slide, therefore, are comparable.

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QUANTITATIVE CYTOCHEMICAL DETERMINATION OF DESOXYRIBONUCLEIC ACID WITH THE FEULGEN NUCLEAL REACTION

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Since the work of Feulgen and Rossenbeck (16) the Feulgen nucleal reaction has been used extensively to demonstrate and localize desoxyribonucleic acid (DNA) in histological preparations. From time to time both the specificity of the reaction and its ability to indicate the site of DNA in the cell have been challenged (5, 29–30). Although these challenges have been of value in pointing out the dangers of uncritical use (*cf.* reference 3), there can be little doubt today that the Feulgen reaction, if used with proper controls, is specific for DNA in histological preparations, and that it shows the site of localization of DNA in the cell (4, 9, 23, 33).

The quantitative use of the Feulgen nucleal reaction as a microchemical tool was investigated by Jorpes (19), Widström (39), Caspersson (6), and Brachet (3). Recently the Feulgen nucleal reaction has been used by several authors for a quantitative determination of DNA in histological preparations (14, 15, 24, 25, 32).

Quantitative cytochemical determinations cannot be relied upon until the methods used have been checked in some way against a standard chemical procedure. In the absence of such a comparison with accepted techniques, cytochemical methods based on light absorption may well be reproducible, but nothing can be stated with regard to their accuracy. In most instances it would be very difficult to devise such a comparison of cytochemical and chemical procedures, but in the case of desoxyribonucleic acid this has become possible with the discovery of the constancy of the DNA content in diploid nuclei of certain vertebrates (2, 22, 37, 38). This constancy permits a comparison of cytochemical determinations on single nuclei with the average value obtained by chemical methods on a large number of isolated nuclei. A series of such values for the DNA content of various vertebrate nuclei (Mirsky and Ris (22) and unpublished data) served as standards in the work to be described in this paper demonstrating that the Feulgen nucleal reaction can be used as a quantitative cytochemical procedure.

Apparatus and Procedure of Measurement

The intensity of the Feulgen reaction in nuclei was measured by using the microscope as a colorimeter. The instrument used has been described and illustrated else-

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where (24). A mercury vapor arc (AH_4) served as light source. With a Wratten filter No. 74 the green line (546 mμ) was isolated and used to illuminate the microscope. In the optic axis, 10 inches from the ocular, the phototube was mounted with a circular diaphragm that limited the area in the image plane. The phototube used was a RCA electron multiplier tube (1 P 28) in connection with the photovolt electronic photometer Model 512. The phototube was mounted interchangeably with a focusing magnifier. A cell structure to be measured was moved into the center of the field, then focussed upon in the image plane with the focussing magnifier, and moved into the center of the image plane-diaphragm. This diaphragm was closed to the size of the area to be measured. Then the focussing magnifier was replaced by the phototube and a reading was taken. The specimen was now moved until an empty area on the slide near it filled the image plane-diaphragm. From this and the first reading the transmission and extinction of the cell structure were calculated. The intensity of the light was adjusted with neutral density filters between light source and microscope.

The amount of absorbing substance can be calculated from the extinction and the dimensions of the structure if the specific extinction of the substance is known. According to the Beer-Lambert law the following equation holds:

$$C_x = \frac{E_x}{E_s D_x} \text{ mg./cc.} \tag{1}$$

where $C_x =$ the unknown concentration in the cell structure, $E_x =$ the extinction of the cell structure, $D_x =$ the thickness of the absorbing layer in centimeters, and E_s the extinction of 1 mg./cc. of the substance with 1 cm. thickness of the absorbing layer as determined for instance in the Beckman spectrophotometer. The total amount of substance present is obtained by multiplying the concentration by the volume of the cell structure. In the simplest case, if the structure is cylindrical, we find:

$$\text{Total amount} = \frac{E_x A D_x}{E_s D_x} \text{ mg./cc.;} \qquad \text{or} \tag{2}$$

$$\frac{E_x A}{E_s} \text{ mg./cc.} \tag{3}$$

where A is the cross-sectional area of the cylinder measured in square centimeters. We see from equation (3) that the thickness of the cylinder measured does not have to be known. This is of great advantage, since accurate thickness measurements with the microscope are very difficult. The cross-sectional area is easily determined from the diameter of the image plane-diaphragm and the magnification used.

In the case of cell nuclei, however, we are not dealing with cylinders, but, in the simplest case, with spheres. The concentration in a sphere can be found by measuring the extinction of a small central cylinder and the diameter of the sphere (Fig. 1 a). But nuclei are rarely perfectly spherical, so that errors in the

measurements of diameters may lead to great errors in the determination of the volume. It would be of great advantage if it were feasible to use the extinction of the cylinder closely circumscribing the sphere (Fig. 1 *b* and Fig. 2). The determination of the total amount of absorbing substance would then become very simple by using equation (3). However, an error is introduced by this method, since the absorbing material is not evenly distributed in the cylinder measured, but concentrated in the sphere within the cylinder. It is shown below how the error thus introduced can be determined empirically. It was found to be within a few per cent and therefore negligible. We are, therefore, justified in the case of nuclei which are nearly spherical in shape to circumscribe with the image plane-diaphragm the maximal cross-sectional area of the nuclei and to apply equation (3) for calculating the total amount of absorbing substance contained in the nuclei.
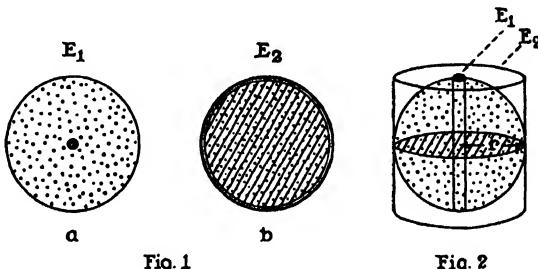
FIGS. 1 and 2. For explanation see text.

For relative measurements it is not necessary to know E_s in equation (3). $E_s A$ is then a convenient comparative value.

Checks of Apparatus and Procedure of Absorption Measurements

Since so many factors influence absorption measurements with the microscope, some of which cannot be evaluated exactly by theoretical considerations alone, it is necessary to check the method against some independent determinations with a standard procedure. In the same way some of the factors influencing absorption measurements were investigated empirically. (For a theoretical discussion of the possibilities and limitations of absorption measurements consult the fundamental paper by Caspersson (7).)

1. A very simple check on the microscope colorimeter will be described first. Lightly colored cellophane (for instance bluish-red, similar in color to the Feulgren reaction) is cut into strips. Three such strips are mounted in clarite on a microscope slide so that they form steps of 1, 2, and 3 layers in thickness. The slide is fixed to a cardboard with a slit, so that it can be fitted into the Beckman spectrophotometer. The extinctions for the 1, 2, and 3 layers are determined using the spectrophotometer with minimal

slit width according to the instructions furnished by National Technical Laboratories. In this way a spectral band width of only 2 to 3 mμ is obtained at the setting for 546 mμ. The extinctions of the cellophane strips are then determined with the microscope colorimeter, at a wave length of 546 mμ (AH$_4$ mercury vapor arc and Wratten filter No. 74). Table I gives the values obtained with a 2 mm. and 4 mm. apochromat (Zeiss) and with the Beckman spectrophotometer. They show that for objects of uniform thickness the microscope colorimeter gives results which are in good agreement with those obtained with the Beckman spectrophotometer.

2. To simulate more closely the conditions encountered in measuring absorption of cell nuclei another check was used which had been described first by Caspersson (7). Sudan IV was extracted for 24 hours with hexane. A component of the dye went into solution. The insoluble part was centrifuged off and the extinction of the dye in hexane was measured in the Beckman spectrophotometer at 546 mμ. The hexane solution was then emulsified in 30 per cent sucrose by shaking in a test tube. The dye is insoluble in sucrose and, with the refractive index of hexane and 30 per cent sucrose

TABLE I

Absorption of Cellophane as Determined in the Beckman Spectrophotometer and with the Microscope (546 mμ)

Cellophane layers	E Beckman	E microscope	
		4 mm. objective	2 mm. objective
1	0.130	0.131	0.125
2	0.265	0.268	0.244
3	0.385	0.377	0.367

almost identical, microscopic absorbing but non-refractile droplets were obtained. A drop of the emulsion was introduced into a cell about 100 μ deep, which was then placed on the microscope stage. Using a small central spot for absorption measurements the extinction at 546 mμ and the diameter of thirty droplets were measured with a 2 mm. and 4 mm. objective. In Fig. 3 the extinction of each droplet is plotted against the diameter. The solid line is calculated from the value obtained with the Beckman spectrophotometer. The average extinction for 1 cm. thickness was then calculated for the thirty measurements and compared with the extinction obtained with the Beckman spectrophotometer (Table II). The measurements on the droplets obtained with the microscope colorimeter are in close agreement with the measurements on the solution with the spectrophotometer.

3. These droplets of Sudan IV in hexane suspended in sucrose also made it possible to determine empirically the error introduced by calculating the amount of absorbing substance in a sphere from the extinction of a cylinder enclosing the sphere according to equation (3). On twenty-five droplets the following measurements were made: (a) the extinction (E_1) using a small central spot was determined at 546 mμ (Figs. 1a and 2); (b) the extinction (E_2) of the cylinder enclosing the same droplet was then measured (Figs. 1 b and 2). The image plane-diaphragm was closed on the maximal cross-

sectional area of the sphere (area $= \pi r^2$). E_1 is proportional to the concentration in the droplet, while E_2 is proportional to the average concentration in the cylinder around the sphere, neglecting the error due to the uneven distribution of absorbing material in the cylinder. Since the volume of the cylinder is $\frac{2}{3}$ of the volume of the
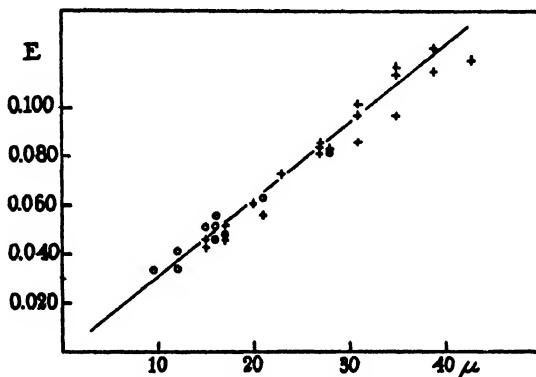
FIG. 3. Absorption of 30 droplets of Sudan IV in hexane, suspended in 30 per cent sucrose, measured with the microscope (+ with 4 mm. objective, O with 2 mm. objective). Extinction plotted against diameter of droplets. The solid line is calculated from the extinction of the same solution of Sudan IV in hexane as determined in the Beckman spectrophotometer.

TABLE II

Absorption of Saturated Solution of Sudan IV in Hexane as Determined in the Beckman Spectrophotometer and on 30 Droplets with the Microscope

Sudan IV in hexane	Beckman E for 1 cm.	Microscope Average E calculated for 1 cm.	Difference
Diluted 50 $\times$	0.615	—	
" 100 $\times$	0.325	—	
Undiluted (calculated)	31.6	30.5 $\pm$ 0.44	3.5 per cent

sphere and the total amount of absorbing material is the same, the average concentration in the cylinder must be $\frac{2}{3}$ of the concentration in the sphere. Consequently

$$\frac{E_1}{E_2} = 1.5$$

The average ratio $\dfrac{E_1}{E_2}$ obtained from measurements on twenty-five nuclei was found to be 1.45. The error due to the uneven distribution of absorbing substance in the cylinder around the droplet is therefore very small and can be neglected. We are thus justified in using the area circumscribing the sphere for absorption measurements and calculating the total amount of absorbing substance according to equation (3).

4. The nuclei one encounters in tissues are rarely exactly spherical. It is therefore sometimes necessary to use an area larger than πr^2 to enclose all nuclear material. This, of course, increases the unevenness of distribution of absorbing material in the space measured. In the following series of measurements an attempt was made to estimate the error introduced by making the area of the image plane-diaphragm larger than the maximum cross-sectional area of the nucleus. The nuclei used were treated as described later in this paper to give an even distribution of DNA and consequently uniform Feulgen staining inside the nucleus. A large nucleus of low DNA concentration (turtle liver nucleus) and a small nucleus with high DNA concentration (turtle erythrocyte nucleus) were measured. First ·the image plane-diaphragm was closed exactly around the nuclear membrane, then the area measured was increased 1.2 times and 1.5 times. For each setting of the image plane-diaphragm ten determinations of E times area were made on the same nucleus. From Table III one sees that with the large and lightly stained nucleus the increase in non-absorbing area around the nucleus did not affect the result. With such nuclei the image plane-diaphragm does

TABLE III

Effect on Absorption Measurements of Uneven Distribution of Absorbing Substance in the Area Measured

Turtle liver nucleus lightly stained				Turtle erythrocyte nucleus densely stained			
Area μ^2		$E \times$ area	n^*	Area μ^2		$E \times$ Area	n
41	1	5.5 ± 0.03	10	20	1	5.2 ± 0.04	10
50	1.2	5.4 ± 0.08	10	23	1.2	4.6 ± 0.03	10
63	1.5	5.6 ± 0.04	10	31	1.6	4.4 ± 0.04	10

$*n$ = number of determinations on the same nucleus. Mean and standard deviation.

not have to fit closely around the nucleus. With the smaller and more densely stained erythrocyte nucleus, however, the increase in non-absorbing area around the nucleus caused a considerable decrease in the product of extinction times area, as is to be expected with the uneven distribution. In measuring small, densely stained nuclei, therefore, even distribution of the absorbing material is essential and the image plane-diaphragm has to fit closely around the outline of the nucleus.

5. The microscope colorimeter differs from the usual colorimeter or spectrophotometer in that the light rays are not parallel through the absorbing material, but they form a definite angle depending on the numerical aperture of the system. The effect of this on absorption measurements was discussed by Caspersson (7) and by Uber (36). The difference in absorption as compared with parallel light is almost impossible to evaluate, since a change in the aperture of the system causes a change in other factors affecting absorption. A decrease in the aperture of the light cone for instance increases the error due to light diffraction and a larger aperture of the light cone increases the error due to scattered light. Therefore, a series of measurements were made to determine empirically the effect of a change in the aperture of the light cone. Ten independent determinations of E times area for a carp liver and erythrocyte nucleus stained with Feulgen were made, with the substage diaphragm set at a nu-

merical aperture of 0.25 and 0.85. The results are given in Table IV. The increase in numerical aperture from 0.25 to 0.85 caused only a negligible change in the measurement. In the following measurements the substage diaphragm was usually set between 0.3 and 0.6 numerical aperture.

6. Glare caused by stray light is an important factor causing errors in absorption measurements. To reduce such scattered light the field diaphragm on the lamp should be closed so that only a small area around the structure to be measured is illuminated. The same opening of the field diaphragm should be used for all measurements that are to be compared.

7. In the method described here the area illuminated on the phototube changes according to the size of the structure measured. Therefore, it must be made certain that the response of the phototube is uniform over the area used. This was checked

TABLE IV

Effect of Numerical Aperture of System on Absorption Measurements with the Microscope

Numerical aperture, condenser	Carp liver nucleus		Carp erythrocyte nucleus	
	$E \times$ area	n^*	$E \times$ area	n
0.25	3.85 ± 0.05	10	3.5 ± 0.02	10
0.85	3.96 ± 0.02	10	3.68 ± 0.02	10

* n = number of determinations on same nucleus.

TABLE V

Uniformity of Response of Phototube for Various Areas Measured

Diameter of image plane-diaphragm..........	4	5	7	8	9	10
Reading on galvanometer.....................	16	25	48	65	80	95

in the following way. The microscope was focussed on a slide which was then moved to an empty area so that the image plane was uniformly illuminated. The image plane-diaphragm was then set for various diameters from 4 to 10 mm. and the response on the galvanometer was read for each diaphragm setting. As we see from Table V the response of the phototube was proportional to the area up to 10 mm. diameter.

The Feulgen Nucleal Reaction

The exact course of the Feulgen nucleal reaction is still not fully understood. But it is generally assumed that the reaction involves a liberation of aldehyde groups through hydrolysis and that these aldehyde groups react with the leuco-basic fuchsin, forming a colored product. This aldehyde-pararosaniline–SO₂ dye has a broad absorption maximum from 550 mμ to 570 mμ (6, 34). Caspersson found that in the test tube the intensity of the color developed is proportional to the amount of DNA within certain limits of concentration (0.02 per cent to 1.5 per cent), but only if the sample consisted of purified DNA and especially

in the absence of proteins. The intensity of color developed by a given sample of DNA varied greatly, depending especially on the pH, time and temperature of hydrolysis, the pH of the staining solution, and the length of time the reaction was allowed to proceed. The reaction was never found to be quantitative, only part of the DNA taking part. Since it was also impossible to completely standardize the reagents, the reaction could only be used in conjunction with a standard treated together with the unknown.

Some of the factors which affect the intensity of the reaction in histological preparations will be discussed now.

TABLE VI

Time of Hydrolysis and Intensity of Feulgen Reaction after Acetic Acid–Alcohol Fixation (Calf Liver)

Time of hydrolysis	$E \times$ area $n^* = 5$
min.	
2	3.1 ± 0.08
3	3.7 ± 0.12
4	3.8 ± 0.07
6	5.2 ± 0.13
8	5.9 ± 0.14
10	5.9 ± 0.17
11	6.1 ± 0.10
12	6.0 ± 0.05
13	6.0 ± 0.06
15	6.1 ± 0.06
20	4.8 ± 0.10

* n = number of nuclei measured.

1. *Time of Hydrolysis.*—The effect of time and temperature of hydrolysis on the intensity of the Feulgen reaction was first investigated by Bauer (1) who found that it depended on the type of fixative used. With certain fixatives, hydrolysis exceeding an optimum decreased the intensity of the color developed while with other fixatives prolonged hydrolysis did not change the maximal color intensity. This was confirmed by Hillary (17) on DNA in agar blocks and plant nuclei and by the absorption measurements of Di Stefano (14, 15).

In the present work two fixatives were used: (a) acetic acid–alcohol (1:3), and (b) formalin (10 per cent and 20 per cent). In the following some measurements on the relation of time of hydrolysis to the intensity of the color after these two fixatives will be reported.

(a) *Fixation with Acetic Acid–Alcohol.*—Calf liver nuclei isolated according to the citric acid method (cf. reference 21) were fixed with acetic acid–alcohol, smeared on a slide, and dried. They were then hydrolyzed for various lengths of time in 1 N HCl at 60°C. and stained in leucobasic fuchsin for 1 hour. After rinsing in SO₂ water three times for 5 minutes each, they were rinsed in tap water for 15 minutes, dehydrated, and mounted in clarite X. A slide treated in the same way except for the leucobasic

fuchsin was mounted in clarite X to determine the blank; *i.e.*, the absorption of un-stained nuclei at 546 mμ. It was found that unstained nuclei are practically invisible in clarite and transmit over 95 per cent of light. The blank was therefore neglected for nuclei mounted in clarite in all the measurements reported below.

The absorption of spherical nuclei was measured as described above. Since we were only interested in relative measurements, E_s in equation (3) does not have to be known and extinction times area was used as a measure of the dye content. The values for extinction times area after various periods of hydrolysis are given in Table VI. There is a broad maximum of color intensity from 8 minutes to 15 minutes of hydrolysis. Longer hydrolysis decreases the intensity of the reaction. In the following 12 minutes of hydrolysis were used whenever the material had been fixed in acetic acid–alcohol.

(b) *Fixation with Formalin.*—Rat liver perfused with 10 per cent formalin and frog liver fixed in 20 per cent formalin were sectioned 10μ and 15μ thick, hydrolyzed for

TABLE VII

Time of Hydrolysis and Intensity of Feulgen Reaction after Fixation in Formalin

Time of hydrolysis	Rat liver nuclei (tetraploid) $E \times$ area ($n = 10$)	Frog liver nuclei $E \times$ area ($n = 10$)
min.		
4	—	8.8 ± 0.11
5	8.1 ± 0.14	—
8	—	12.0 ± 0.2
10	10.3 ± 0.04	12.3 ± 0.17
12	—	12.4 ± 0.14
13	10.9 ± 0.17	13.8 ± 0.17
15	12.1 ± 0.1	13.8 ± 0.16
20	11.8 ± 0.14	14.2 ± 0.20
25	12.2 ± 0.14	13.9 ± 0.14

various lengths of time, stained for 1 hour in leucobasic fuchsin, and mounted in clarite. The absorption of spherical nuclei (in medium size rat nuclei = tetraploid, see below) was measured and extinction times area calculated. Table VII shows the effect of time of hydrolysis on the intensity of color produced. A maximum intensity was reached after 13 minutes of hydrolysis and continuous hydrolysis up to 25 minutes did not decrease the intensity of the reaction. In the measurements reported below 15 minutes of hydrolysis was used after formalin fixation.

(c) *Comparison of Maximal Color Intensity after Acetic Acid–Alcohol and Formalin Fixation.*—Calf liver nuclei fixed in acetic acid–alcohol and with formalin were hydro-lyzed for 15 minutes and stained together. As Table VIII shows the maximal intensity of the reaction is the same after the two fixatives.

2. *Time of Staining.*—Caspersson (6) found that in the test tube the color intensity varied with the time the reaction was allowed to proceed. A maximum was reached after 3 to 4 hours after which the intensity decreased rapidly. To determine the influence of time of staining in histological preparations, calf liver fixed in acetic acid–alcohol was sectioned 10μ thick, hydrolyzed for 12 minutes, and stained for

various periods of time. The results are shown in Table IX. The maximum color intensity of the nuclei is reached within one-half hour. Prolonged immersion in leucobasic fuchsin decreases the intensity of the reaction, probably due to continuous hydrolysis in the acid solution.

3. *Method of Preparation of the Leucobasic Fuchsin.*—Feulgen and Rossenbeck (16) described two methods for the preparation of the leucobasic fuchsin. It can be prepared by bubbling SO_2 gas through the basic fuchsin solution until it is decolorized or by adding sodium metabisulfite (or potassium metabisulfite (*cf.* reference 12)) and

TABLE VIII

Maximal Intensity of Feulgen Reaction in Calf Liver Nuclei after Acetic Acid–Alcohol and Formalin Fixation

Fixation	$E \times$ area ($n = 5$)
Acetic acid–alcohol....................................	6.1 ± 0.06
Formalin 20 per cent.................................	6.1 ± 0.1

TABLE IX

Effect of Time of Staining on the Intensity of the Feulgen Reaction in Calf Liver Nuclei (Fixation Acetic Acid–Alcohol)

Time of staining in leucobasic fuchsin....	30 min.	1 hr.	1 hr., 30 min.	24 hrs.
$E \times$ area ($n = 5$)	6.8 ± 0.1	6.5 ± 0.1	6.9 ± 0.1	4.7 ± 0.1

TABLE X

Method of Preparation of Leucobasic Fuchsin and Intensity of Feulgen Reaction. (Mouse Liver Diploid Nuclei; Fixation Acetic Acid–Alcohol)

Preparation of leucobasic fuchsin	$E \times$ area $n = 5$
Rafalko (1946)	5.1 ± 0.17
Coleman (1938)	5.0 ± 0.17

N HCl to the basic fuchsin solution. Since the pH of the reagent influences the intensity of color produced and it is very difficult to control this pH with the first method, Feulgen and Rossenbeck (16) and later Caspersson (6) recommended the second method of preparation. Recently Rafalko (26) claimed that the reagent prepared by bubbling SO_2 gas through the basic fuchsin produced a more intense coloring of nuclear constituents. To compare the color intensity produced by the two types of reagents mouse liver sections fixed in acetic acid–alcohol were stained in the two reagents and the absorption of the smallest nuclei present was measured. The results are presented in Table X. The intensity of color produced was the same with both reagents. Actually, if the excess SO_2 in the reagent prepared according to Rafalko was not removed by letting it stand exposed to air for several hours, the intensity of color produced was less than with the reagent prepared with metabisulfite.

4. *Fading of the Feulgen Reaction.*—The nuclei were always measured within 24 hours after the treatment with leucobasic fuchsin. One slide was measured again after 4½ months and the same result was obtained as right after staining. The intensity of the Feulgen stain in microscopic preparations therefore does not change within several months at least (*cf.* reference 17).

Measurements on Various Nuclei of Known DNA Content

1. *Liver Nuclei.*—In the course of a study on the DNA content of nuclei in the tissues of vertebrates, the DNA content of isolated liver nuclei of a number of vertebrates had been determined (Mirsky and Ris (22), and unpublished data). Some of these nuclei were fixed in acetic acid–alcohol, smeared on slides, and stained by the Feulgen procedure. The relative intensity of the reaction was then measured with the microscope colorimeter and compared with the DNA content as determined by chemical methods on a known number of nuclei. The results are shown in Table XI.

TABLE XI

Intensity of Feulgen Reaction and DNA Content of Isolated Nuclei of Shad (Alosa sapidissima), Chicken, and Calf Liver, the Calf Liver Used as Standard. Fixation Acetic Acid–Alcohol

	Shad	Chicken	Calf
$E \times$ area	1.20 ± 0.04 ($n = 5$)	1.57 ± 0.05 ($n = 10$)	4.3 ± 0.17 ($n = 5$)
DNA per nucleus mg. $\times 10^{-9}$ chemical determination	2.0	2.4	6.5
DNA per nucleus calculated from Feulgen calf as standard	1.8	2.4	6.5

In the first row the values for extinction times area calculated from the absorption measurements are given. In the second row the DNA content of these nuclei is shown, and in the third row the DNA content of the same nuclei calculated from the Feulgen reaction by using the calf liver nucleus as standard. These values agree closely with those obtained by standard chemical determinations. It is therefore possible to determine the DNA content of nuclei with the Feulgen reaction, if nuclei of known DNA content are used as standard, treated together with the unknown.

Another series of measurements were made on sections of livers of five different fish. The DNA content in nuclei of these fish had been determined chemically on erythrocytes. It was shown that in fish the DNA content of erythrocyte nuclei and liver nuclei is the same (Mirsky and Ris (22), and unpublished data). Small pieces of liver were fixed in acetic acid–alcohol, sectioned at 10μ, hydrolyzed 12 minutes, and stained in leucobasic fuchsin for 1 hour. Sections of shark liver were placed on every slide as standard. In making absorption measurements on nuclei in sections one must, of course, make certain that the entire nucleus is within the section. Table XII shows the results for these nuclei. In the third row again the DNA content of the nuclei calculated from the Feulgen reaction by using the shark liver nuclei as

standard is given. By comparison with the second row we see that the relative values calculated from the Feulgen reaction agree with the microchemical determinations within about 10 per cent.

Since we used both smears of isolated nuclei and nuclei in sections, measurements were made to find out whether any DNA was lost during isolation of the nuclei or whether the DNA content as determined by the Feulgen reaction was the same in

TABLE XII

Intensity of Feulgen Reaction and DNA Content of Liver Nuclei of Yellow Tail (Ocyurus chrysurus), Red Hind (Epinephelus guttatus), Houndfish (Tylosurus acus), Yellow Grunt (Haemulon flavolineatum) and Dusky Shark (Carcharias obscurus), with the Shark Used as Standard. Fixation Acetic Acid–Alcohol

	Yellow tail	Red hind	Houndfish	Yellow grunt	Dusky shark
$E \times$ area ($n = 5$)	0.95 ± 0.03	0.76 ± 0.01	1.05 ± 0.04	0.55 ± 0.01	—
Shark	2.20 ± 0.03	2.20 ± 0.1	2.24 ± 0.05	2.28 ± 0.03	—
DNA per nucleus mg. $\times 10^{-9}$ chemical determination	2.1	2.1	2.2	1.2	5.5
DNA per nucleus calculated from Feulgen shark as standard	2.4	1.9	2.5	1.3	5.5

TABLE XIII

Comparison of the Intensity of Feulgen Reaction in Isolated Calf Liver Nuclei and Calf Liver Nuclei in Sections. Fixation Acetic Acid–Alcohol

	$E \times$ area $n = 10$
Isolated citric nuclei....................................	6.38 ± 0.14
Nuclei in sections (10μ)................................	6.44 ± 0.11

isolated nuclei and in nuclei fixed *in situ*. Calf liver fixed in acetic acid–alcohol was sectioned at 10μ. On the same slide calf liver citric nuclei which had been fixed in acetic acid–alcohol, were smeared and fixed on the slide by drying. The slide was then stained by the Feulgen procedure and the absorption of the nuclei at 546 mμ determined. From Table XIII it can be seen that the intensity of the Feulgen reaction is the same in isolated nuclei and in nuclei fixed *in situ*. Accordingly no DNA is lost during the isolation of these nuclei in citric acid.

In many tissues of mammals the nuclei are not all the same size. In a number of investigations by many authors it was found that in general the volumes of these nuclei form ratios of 1:2:4 etc. By stimulating such nuclei to divide it was demonstrated that the larger nuclei are polyploid, that with each step of

increase in volume the chromosome number had been doubled (27, 35). This polyploidy is especially pronounced in the liver of rodents. Rat liver, fixed in 10 per cent formalin by perfusion and sectioned at 15μ, was used to find out whether the intensity of the Feulgen reaction was proportional to the number of chromosome sets in these polyploid nuclei. In rat liver three sizes of nuclei are common, corresponding presumably to a diploid, tetraploid, and octoploid number of chromosomes. In Table XIV the values for extinction times area obtained by absorption measurements on these nuclei stained with Feulgen are given for the three sizes of nuclei. The ratios of intensity of the Feulgen reaction are very close to 1:2:4 and are therefore proportional to the ratios of chromosome number.

TABLE XIV

Size of Nuclei (Polyploidy) and Intensity of Feulgen Reaction in Rat Liver. Fixation 10 Per Cent Formalin

Size of nuclei	$E \times area$ $n = 10$	Ratio
Smallest nuclei................	5.5 ± 0.1	1
Medium sized nuclei..................................	10.4 ± 0.1	1.9
Largest nuclei...	19.9 ± 0.2	3.6

TABLE XV

Intensity of Feulgen Reaction in Hepatic and Erythrocyte Nuclei of the Dusky Shark (Carcharias obscurus) and the Chicken. Fixation Acetic Acid–Alcohol

	Liver nuclei $E \times area$ $n = 10$	Erythrocyte nuclei $E \times area$ $n = 10$	Ratio
			per cent
Dusky shark........................	3.96 ± 0.13	2.70 ± 0.17	68
Chicken...........................	1.57 ± 0.05	1.04 ± 0.02	66

2. Measurements on Nuclei of Different Tissues.—So far we have compared only nuclei of one tissue and with similar structure, distribution of chromatin, and DNA concentration. Does the proportionality of the Feulgen reaction with DNA content hold also for nuclei with entirely different structure and DNA concentration?

In the liver of lower vertebrates we find two types of nuclei which are convenient for the study of this question, namely the relatively lightly staining large hepatic nuclei and the much smaller and very densely staining nuclei of the erythrocytes. The intensity of the Feulgen reaction was measured for hepatic and erythrocyte nuclei in sections of shark liver and in smears of isolated nuclei from chicken liver, both after fixation in acetic acid–alcohol. The results are shown in Table XV. We find that in both shark and chicken the intensity of the Feulgen reaction as determined with the microscope colorimeter

is more than 30 per cent less in erythrocyte nuclei than in hepatic nuclei. However, it is known from chemical determinations that the DNA content of erythrocyte and hepatic nuclei is the same (Mirsky and Ris (22), and unpublished data). It seems, therefore, that it is not possible to determine the relative DNA content of nuclei from the intensity of the Feulgen reaction if these nuclei differ markedly in structure.

Considering a possible cause for this disproportionality one may think first of the great difference in the distribution of chromatin in these nuclei fixed with acetic acid–alcohol. Now it was found·that the distribution of chromatin in nuclei depends very much on the state of the nucleus before fixation and on the type of fixative used (28). In the living nucleus and in the nucleus isolated in non-electrolytes (for instance sucrose) the chromatin is more or less uniformly distributed, since the chromosomes are greatly swollen and fill the entire nuclear space except for the nucleoli. Nuclei with the chromatin in this extended state can be fixed for instance with formalin without changing the distribution of the chromatin. After treatment with the Feulgen reagents the nuclei are then stained quite uniformly, only the nucleoli remaining unstained. In tissues removed from the animal the nuclei were always found to be in the condensed state no matter how quickly they were fixed. But the chromatin in these nuclei could be extended by isolating them in sucrose. No DNA was lost even if they remained in sucrose overnight in the cold (28).

The most uniformly stained nuclei were obtained in· the following manner. The tissue was cut into small pieces in the cold and then homogenized in 30 per cent sucrose with an Elvehjem-Potter homogenizer. This was continued until microscopic examination showed most cells fragmented and the nuclei isolated. When there was much pigment present in the tissue, the pigment granules were removed by differential centrifugation according to the procedure of Hogeboom, Schneider, and Pallade (18). A drop of the homogenate was then smeared on a microscope slide and inverted on 20 per cent formalin in a Petri dish. After 10 minutes of fixation the slides were washed for 2 to 3 hours in running water to remove all traces of formaldehyde. After hydrolysis for 15 minutes in 1 N HCl at 60°C. the slides were stained with the leucobasic fuchsin for 1 hour, rinsed in SO₂ water in three changes for 5 minutes each, and mounted in clarite. The nuclei were now found to be stained uniformly by the Feulgen reaction. Figs. 4 to 9 show hepatic and erythrocyte nuclei prepared in this way from carp, chicken, and green turtle (*Chelonia mydas*).

Spherical nuclei were then selected and the intensity of the Feulgen reaction determined with the microscope colorimeter in both hepatic and erythrocyte nuclei. In measuring the absorption of erythrocyte nuclei it was made certain that the image plane-diaphragm fitted closely around the nuclei and no empty area was enclosed, since, as was discussed above, large errors are introduced by that. The results of these measurements are given in Table XVI. We see that with the much more even distribution of the chromatin in the nuclei the difference in the values extinction times area for the two types of nuclei has become negligible. The values obtained with this·
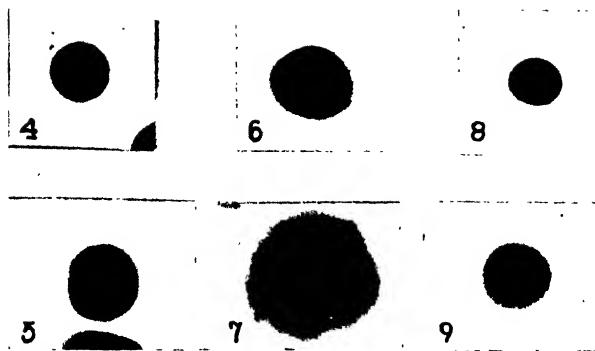
FIGS. 4 to 9. Erythrocyte and hepatic nuclei isolated in sucrose, fixed with 20 per cent formalin, and stained with Feulgen. Note the uniform distribution of the DNA. Figs. 4 and 5, erythrocyte and hepatic nuclei of carp, Figs. 6 and 7 from green turtle, Figs. 8 and 9 from chicken. (2 mm. Zeiss apochromat, 15 x ocular. × 2400).

TABLE XVI

Intensity of Feulgen Reaction in Hepatic and Erythrocyte Nuclei with Even Distribution of DNA. Nuclei Isolated in 30 Per cent Sucrose and Fixed in 20 Per cent Formalin

	Liver nuclei		Erythrocyte nuclei	
	$E \times$ area	n	$E \times$ area	n
Carp	3.8 ± 0.05	20	3.4 ± 0.04	20
	3.85 ± 0.05	10	3.5 ± 0.02	10
Green turtle	5.3 ± 0.1	16	5.3 ± 0.1	14
(*Chelonia mydas*)	5.5 ± 0.03	10	5.2 ± 0.04	10
Chicken	2.9 ± 0.04	20	2.6 ± 0.05	20

TABLE XVII

Intensity of Feulgen Reaction and DNA Content in Liver Nuclei with Even Distribution of DNA. Nuclei Isolated in Sucrose and Fixed in 20 Per Cent Formalin

	Carp	Bull frog	Green turtle	Chicken
$E \times$ area $n = 20$	3.8 ± 0.05	18.3 ± 0.02	5.3 ± 0.1	2.9 ± 0.04
DNA per nucleus mg. $\times 10^{-9}$ chemical determination	3.3	15.7	5.1	2.4
DNA calculated from Feulgen using carp as standard	3.3	15.8	4.6	2.5
DNA calculated according to Di Stefano (1948) mg. $\times 10^{-9}$	0.8	4.0	1.1	0.5

method from absorption measurements on Feulgen-stained nuclei are proportional to the DNA content even for nuclei with greatly different structure and DNA concentration.

Table XVII shows measurements on Feulgen-stained liver nuclei in the extended state from four different vertebrates. In the third row we find again the DNA content of these nuclei as calculated from the intensity of the Feulgen reaction, using the carp liver nucleus as standard. A comparison with the second row shows that the relative amounts of DNA for these nuclei as determined from the Feulgen reaction agree closely with the microchemical analysis of isolated citric nuclei.

Absolute Quantitative Determination of DNA from the Feulgen Nucleal Reaction

Di Stefano (14, 15) and Pollister and Leuchtenberger (25) have calculated the absolute amounts of DNA per nucleus from absorption measurements on Feulgen-stained nuclei, by assuming with Wieland and Scheuing (40) that one molecule of basic fuchsin combines with two aldehyde groups liberated by the hydrolysis from two purine nucleotides, and that all the reactions involved proceed to completion. The specific extinction of the basic fuchsin -SO_2-aldehyde molecule was determined on leucobasic fuchsin colorized with formaldehyde, assuming that it is identical with the specific extinction of the basic fuchsin -SO_2-DNA-aldehyde.

The findings of Caspersson (6) on the Feulgen reaction *in vitro* and the recent work of Chong-Fu Li and Stacey (9) make it most unlikely that the Feulgen reaction on histological material is a quantitative process as assumed by those authors.

If we calculate the amount of DNA per nucleus from the intensity of the Feulgen reaction in the liver nuclei shown in Table XVII, we find that the result is indeed quite different from the values obtained by the standard microchemical analysis. The values calculated according to Di Stefano are about 25 per cent of the values obtained from microchemical analysis (Table XVII, fourth row).

It follows that the intensity of the Feulgen nucleal reaction can only be used for relative determinations of DNA in cellular structures. If absolute values are desired, standard nuclei of known DNA content must be treated together with the unknown material.

DISCUSSION

If the Feulgen nucleal reaction is to be used for a quantitative cytochemical determination of desoxyribonucleic acid (DNA) it must be shown, (1) that the reaction is specific for DNA, (2) that the colored product is not diffusible but

remains at the site of DNA in the cell, and (3) that the intensity of color produced is proportional to the amount of DNA present.

There seems to be general agreement today that in fixed histological preparations there is no material other than DNA present that can give a true nucleal reaction. With true nucleal reaction is meant the development of color with leucobasic fuchsin after, but not before hydrolysis in N HCl.

Stedman and Stedman (30, 31) claimed that the fuchsin-SO_2-DNA product of the Feulgen reaction was diffusible and they therefore challenged the possibility of localizing the site of DNA in the cell with the Feulgen reaction. Furthermore, it was shown that chromosomes are stained by leucobasic fuchsin recolorized by hydrolyzed DNA (5, 10, 11, 13, 30). Danielli, however, made it clear that this staining with the developed nucleal stain was essentially different from the Feulgen reaction in that not only the chromosomes but also the cytoplasm was stained. It is possible that under certain conditions a diffusible fuchsin-SO_2-DNA product is formed that acts as a basic dye, but the recent work of Chong-Fu Li and Stacey (9) and Overend and Stacey (23) makes it most unlikely that this occurs under conditions of a properly executed Feulgen reaction on histological material. This conclusion is supported also by a comparison of the Feulgen reaction with the sites of absorption at 2600 Å (8). We can, therefore, safely assume that the Feulgen nucleal stain in histological preparations reveals the site of localization of DNA.

The work of Widström (39) and Caspersson (6) established that under certain conditions the intensity of the nucleal reaction was proportional to the concentration of DNA. This was true in concentrations from 0.02 per cent to 1.5 per cent and for purified DNA, but not in the presence of proteins. The reaction did not proceed quantitatively, only part of the DNA taking part.

In the present paper it was shown that in nuclei with a DNA concentration up to a few per cent the intensity of the color is proportional to the DNA content. This was demonstrated by comparing the absorption of nuclei at 546 mμ with the DNA per nucleus as determined by independent chemical determination on a known number of nuclei. This comparison was made possible by the remarkable fact that diploid nuclei within one species contain the same amount of DNA (2, 22, 37, 38). It was thus possible to compare a single nucleus with the average of a large number of nuclei (in the absence of polyploidy).

The possibility of a quantitative use of the Feulgen reaction was recently questioned by Lessler (20). He prepared various concentrations of DNA in gelatin, placed a drop on a slide, and applied the Feulgen reaction. The intensity of the color developed was judged by eye. In this way he found that concentrations above 0.15 per cent did not yield greater intensity of color. It is of course impossible to compare light absorption of densely stained preparations in this manner, and the observations of Lessler are therefore no valid criticism of a quantitative use of the Feulgen reaction.

In histological preparations as in the test tube the nucleal reaction does not proceed quantitatively. It is therefore not possible to calculate the absolute amounts of DNA from the amounts of basic fuchsin present as was done by Di Stefano (14, 15) and Pollister and Leuchtenberger (25). Only relative values can be obtained from the intensity of the nucleal reaction. For absolute determinations nuclei of known DNA content must be treated together with the unknown to serve as a standard.

The nucleal reaction executed according to a standard procedure yields reproducible results. But even so it is advisable to use nuclei of known DNA content as standard either on the same slide or on another slide treated together with the unknown material.

Determinations of the color intensity after various periods of hydrolysis on material fixed with acetic acid–alcohol and with formalin confirmed in general the observations of Bauer (1), Hillary (17), and Di Stefano (15), that the relation of hydrolysis to color intensity depends on the type of fixation. In the case of acetic acid–alcohol an optimum curve was found in agreement with the authors cited. However, the curve showed a broad maximum after hydrolysis for 8 to 15 minutes and not a narrow peak after 12 minutes as reported by Di Stefano (14, 15). After formalin fixation a maximum intensity of the Feulgen reaction was reached with a 13 minute hydrolysis in both rat and frog liver. Longer hydrolysis, up to 25 minutes, did not decrease the intensity of the reaction. This is in marked contrast to the result obtained by Hillary (17), who found that after formalin fixation the intensity of color decreased with hydrolysis beyond a narrow optimum just as after acetic acid–alcohol fixation. Only after chromic acid did he find persistent maximal intensity with prolonged hydrolysis. The data of Hillary were based primarily on experiments with DNA in agar blocks and it is likely that under these conditions DNA behaves differently than when combined with nuclear proteins.

The effect of time of staining on the intensity of the nucleal reaction has not been studied quantitatively before. Our measurements have shown that the intensity of color is the same after staining for one-half up to several hours. Prolonged sojourn in leucobasic fuchsin, however, decreases the intensity of the reaction.

The concentration of the fuchsin–SO_2–DNA product in the nuclei was determined by absorption measurements with the microscope at a wave length of 546 mμ. The possibilities and limitations of absorption measurements on cellular structures with the microscope were first investigated by Caspersson (6), who discussed in detail the conditions under which such measurements are feasible as well as the major sources of error. A simplified apparatus and method of measurement were described by Pollister and Ris (24). The same apparatus and procedure were used in the present paper. The accuracy of the method and the magnitude of the errors caused by certain simplifying assumptions were

determined in a series of measurements. It was found that absorption measurements with the microscope on cellophane strips and on droplets of Sudan IV in hexane agreed within a few per cent with determinations on the same material with the Beckman spectrophotometer. Furthermore, it was shown that the errors due to the simplifying assumptions were so small that they could be neglected.

The non-homogeneity of distribution of absorbing material in cellular structures is one of the main sources of error, especially in absolute absorption measurements. The chromatin in nuclei for instance is distributed in fibers and clumps after the usual methods of preparation. Relative measurements on nuclei of similar structure are possible despite this uneven distribution with an error of about 10 per cent as was shown in this paper for various liver nuclei. However, if nuclei of different structure and DNA concentration were compared an error of over 30 per cent was found. Fortunately, this uneven distribution of DNA in the nucleus can be avoided. In the living nucleus DNA is distributed homogeneously and it can be fixed in this condition so that nuclei become uniformly stained by the Feulgen reagents (28). Absorption measurements on such nuclei were found to agree within 10 per cent even if the DNA concentration varied as much as in hepatic and erythrocyte nuclei of lower vertebrates.

In conclusion it can be said that with the apparatus described, absorption measurements on microscopic structures can be made with an acuracy of a few per cent and that relative determinations of the DNA content of nuclei with the Feulgen reaction yield results that agree within 10 per cent with the values obtained from determinations with standard microchemical methods.

SUMMARY

The possibility of using the Feulgen nucleal reaction for a quantitative cytochemical estimation of desoxyribonucleic acid (DNA) was investigated. The intensity of the reaction in nuclei was determined by absorption measurements with the microscope. The accuracy of such measurements was tested by comparison with measurements on the same material with a Beckman spectrophotometer. The values obtained with the microscope agreed within a few per cent with those obtained with the Beckman spectrophotometer. Furthermore, the errors introduced by uneven distribution of absorbing material, by variations in the numerical aperture of the system, and by variation in the area used on the phototube were investigated empirically.

The following variables were studied with regard to their effect on the intensity of the Feulgen reaction: type of fixation, time of hydrolysis after acetic acid–alcohol and formalin fixation, time of staining in leucobasic fuchsin, method of preparation of leucobasic fuchsin.

The intensity of the Feulgen reaction in liver and erythrocyte nuclei of various vertebrates, fixed in acetic acid–alcohol, was then compared with the DNA

content of these nuclei as determined by chemical analysis on a known number of nuclei. The intensity of the reaction was found to be proportional to the DNA content of the nuclei, if nuclei of similar structure and DNA concentration were compared. In nuclei of different structure and DNA concentration (*i.e.* liver and erythrocyte nuclei), fixed in acetic acid–alcohol, the intensity of the Feulgen reaction was, however, not proportional to the DNA content. This difficulty was overcome by isolating nuclei in sucrose and by fixing them in formalin. Uniform distribution of DNA and therefore uniform coloring after the Feulgen reaction were thus obtained. In such nuclei with uniform distribution of absorbing material the Feulgen reaction was found to be proportional to the DNA content of nuclei, even if they differed greatly in their DNA concentration.

The Feulgen nucleal reaction is not quantitative in an absolute sense. For absolute determinations nuclei of known DNA content must be treated together with the unknown material to serve as standard.

From these data it therefore appears possible to determine cytochemically relative amounts of DNA in cellular structures by measuring their absorption after treatment with the Feulgen nucleal reaction.

Note Added to Page Proof.—In addition to the papers on quantitative cytochemical applications of the Feulgen nucleal reaction by Di Stefano (14) and by Pollister and Leuchtenberger (25) which have been referred to in this paper there has recently appeared a paper by Schrader and Leuchtenberger (*Proc. Nat. Acad. Sc.*, 1949, **35**, 464). In this case the authors have made cytochemical measurements by the procedure used previously (14, 25) on cells of different tissues of *Tradescantia* and consider that the "DNA values are relative and not absolute." From the experiments reported in the present paper it can be seen that when measurements on nuclei of certain different cell types are made by a procedure much the same as that used by Schrader and Leuchtenberger widely different values are obtained (Table XV), although when the measurements are made on nuclei in which the DNA has been more uniformly dispersed essentially the same values are found (Table XVI). It is, therefore, unlikely that the determinations of Schrader and Leuchtenberger have a relative quantitative validity. There has indeed been no cytochemical procedure for nucleic acid determination by the Feulgen nucleal reaction for which evidence has been given indicating either absolute or relative quantitative validity.

If the possibilities of error in the measurements of Schrader and Leuchtenberger are recognized, their measurements do not warrant their conclusion: "The unavoidable conclusion is that the amount of DNA carried in a given chromosome may vary in different tissues."

This very conclusion is, however, supported by determinations of DNA given in a paper (Mirsky and Ris, 22) to which Schrader and Leuchtenberger refer, but incorrectly. In referring to this paper Schrader and Leuchtenberger say, "Working on tissues in vertebrates, they arrived at the conclusion that all the diploid cells of an organism carry identical amounts of DNA," and, "Contrary to the findings of several

workers for the tissues of vertebrates, the nuclei in different tissues of the plant *Tradescantia* carry different amounts of DNA." Now what Mirsky and Ris (22) actually said with respect to diploid nuclei was that in certain vertebrates (in some fish and amphibia and in a reptile and a bird) the quantity of DNA in the nucleus of an erythrocyte is the same as in the nucleus of a liver cell of the same species; and it may be noted that these results are nicely confirmed in the present paper. In presenting their data on the DNA contents of the nuclei of cattle, Mirsky and Ris (22) said, "It will be seen that there are some variations from the simple relationships found in other vertebrates." This sentence appears under a table showing how the DNA content per nucleus varies in different diploid nuclei of cattle.

It should be said that Schrader and Leuchtenberger in the above quotations were referring to papers by Boivin and the Vendrelys as well as to the paper by Mirsky and Ris, and possibly this has made some difficulty in quoting the various authors correctly.

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METHYL GREEN-PYRONIN

I. BASIS OF SELECTIVE STAINING OF NUCLEIC ACIDS

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PLATE 1

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Largely as the result of Brachet's investigations (1), it has become appreciated that the differential staining of tissues with the methyl green–pyronin mixture of Unna and Pappenheim is determined by their nucleic acid components. Thus, the green staining of nuclei was demonstrated to be due to their content of desoxyribonucleic acid, and the red staining of the cytoplasm to be determined by the ribonucleic acid content. It is the purpose of this paper to report some observations on the nature of this phenomenon of differential staining of similar acid substrates by two basic dyes.

In tissues, desoxyribonucleic acid apparently occurs as a much higher polymer than ribonucleic acid (2). It appeared to us that this difference in the degree of polymerization might be the determining factor in the differential staining with methyl green–pyronin. It is the purpose of this paper to present observations on the differences in staining between highly polymerized and depolymerized desoxyribonucleic acids. As will be seen, these tend to confirm the hypothesis that the affinity of nucleic acids for methyl green and pyronin is a function of their state of polymerization: methyl green stains preferentially highly polymerized nucleic acid, while pyronin has a special affinity for low polymers (depolymerized desoxyribonucleic acid and ribonucleic acid).

EXPERIMENTAL

Nucleic Acids and Nucleoproteins

1. **Desoxyribonucleohistone (DNH)** was prepared from calf thymus chromosomes by extraction with 1 M NaCl (3). The viscous solution was centrifuged at high speed to remove "residual chromosomes" and nucleoli. The slightly opalescent viscous supernate was stored in the cold with toluene as preservative. The phosphorus analysis of this solution was 0.27 mg./cc.

2. **Desoxyribonucleic acid (DNA)[1]** was prepared from the DNH solution by repeated mixing in a Waring blendor with chloroform-octyl alcohol (4) to remove

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[1] Prepared by Dr. A. E. Mirsky of The Rockefeller Institute for Medical Research.

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the histone, followed by precipitation of the DNA from the clear viscous aqueous solution with alcohol, resolution in water, and reprecipitation in alcohol. The final white, fibrous precipitate was wound about a glass rod, squeezed dry, and dried in a desiccator at room temperature. This material swells and then dissolves in water to form clear, colorless, viscous solutions which give a pure nucleic acid absorption spectrum. The criteria for high polymerization of this material are based on ultracentrifugation, high viscosity of solutions, and fibrous character of the precipitates. A 1 mg./cc. solution was found to have a phosphorus content of 0.085 mg./cc.

3. Ribonucleic acid (RNA) was purchased from Schwarz Laboratories, Inc. The white granular material dissolved in water on the addition of a little NaOH to bring the pH to between 6 and 7. The solutions were colorless and not viscous. They gave a pure nucleic acid absorption spectrum. A 1 mg./cc. solution contained 0.076 mg. P/cc.

4. Depolymerized DNA was prepared from a solution of DNA by the addition of desoxyribonucleo-depolymerase (5). To 15 cc. of a 1 mg./cc. solution of DNA, 0.8 cc. 0.15 M NaHCO₃ was added to adjust the pH to 7.5, then 3 cc. 0.1 mg./cc. gelatin, 5 cc. 0.015 M MgSO₄, and 2 cc. 4 mg./cc. depolymerase. The solution was allowed to stand overnight at room temperature, then stored in the refrigerator with toluene as preservative. The resultant solution is clear, colorless, not at all viscous. The addition of lanthanum salts, alcohol, or dilute acid results in a granular precipitate as for RNA instead of the fibrous precipitate obtained with DNH and DNA.

5. Ribonucleoprotein (RNP) was prepared from calf thymus as described by Mirsky and Pollister (6). The solution was stored in the cold with toluene as preservative. Its P content was 0.10 mg./cc.

6. Tobacco mosaic virus was provided through the courtesy of Dr. G. Oster of The Rockefeller Institute for Medical Research, Princeton, New Jersey. The solution contained 0.014 mg. P/cc.

Stains

A brief description of the source and characteristics of the stains used follows:

1. The methyl green used in these experiments was labelled "National Aniline Corp., C.I. No. 684, certified for general histological staining, certification No. NC3." The commercial dye was made up to 0.25 per cent in 0.2 M pH 4.1 acetate buffer. In order to remove the considerable amount of crystal violet present in the commercial dye, the solution was extracted with successive portions of chloroform until the chloroform extracts were colorless. The absorption spectrum of this solution, suitably diluted with buffer, was determined in the Beckman spectrophotometer (Text-fig. 1). By comparison with the extinction coefficient at the maximum (635 mμ) of a known solution of the perchlorate (prepared by the precipitation of methyl green perchlorate from the purified solution of the dye described above by the addition of a few drops of a dilute aqueous solution of sodium perchlorate, washed with cold water, dried at 106°C., theoretical N 7.16 per cent, found 6.99 ± 0.2 per cent (Dumas)), this solution was found to contain 51.4 per cent of the labelled concentration of dye (ϵ_{635} (molar extinction coefficient at 635 mμ) = 74,400). This was consistent with the nitrogen content of the solution (theoretical N for 0.25 per cent solution 0.220 mg./cc., found 0.128 mg./cc., corresponding to a dye content of 55.7 per cent).
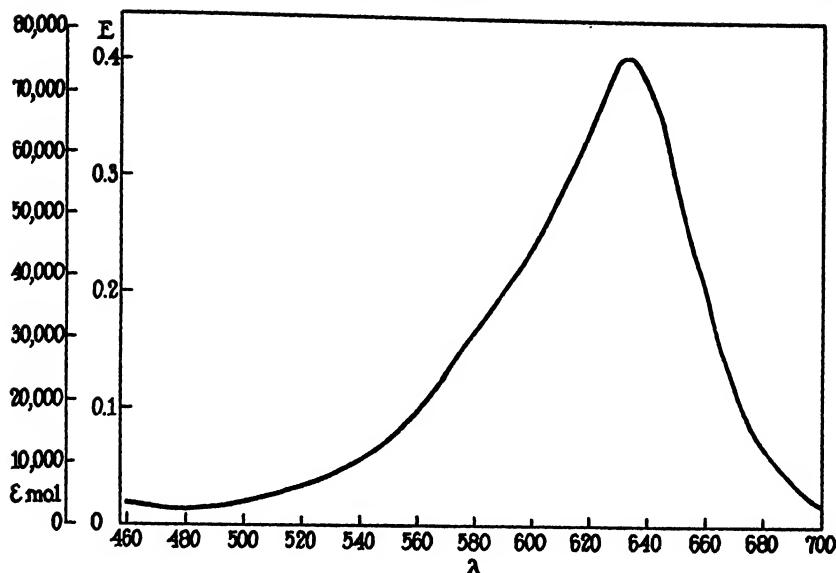
TEXT-FIG. 1. Absorption spectrum of methyl green perchlorate, 0.000323 per cent in pH 4.1 acetate buffer.
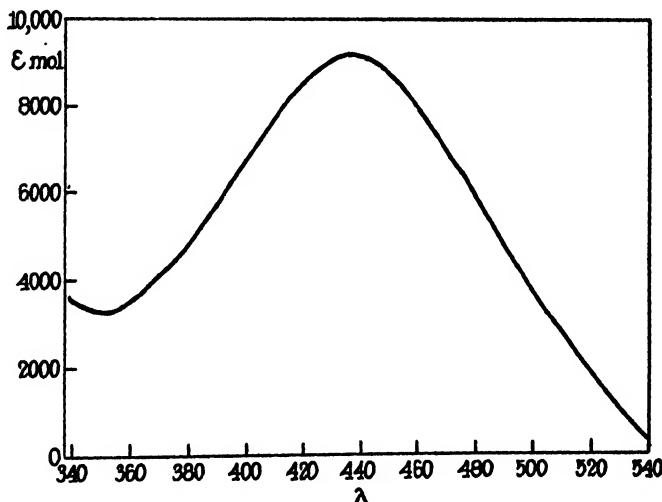
TEXT-FIG. 2. Absorption spectrum of methyl green in concentrated hydrochloric acid (0.00025 per cent and 0.0025 per cent).

Solutions of methyl green in concentrated HCl are brown with an absorption maximum at 440 mμ (Text-fig. 2). This brown solution follows Beer's and Lambert's laws, and has an ϵ_{440} of 8920.

In staining experiments with this dye, the use of a buffer at about pH 4.1 is essential. It was found that above pH 5.0 and below pH 3.5, solutions of the dye are very unstable to light. At pH 4.1, solutions stored in brown bottles for several months showed a reduction in extinction coefficients of less than 10 per cent, and no loss of absorption could be detected during the course of the experiments. It should be noted also that alcoholic solutions of the dye fade exceedingly rapidly. Solutions of the dye in the buffer follow Beer's and Lambert's laws, and the absorption spectrum is independent of the concentration.

2. Several samples of pyronin were investigated. All gave stable solutions which, unlike methyl green, showed no tendency to fade over the pH range 2.9 to 7.5. How-
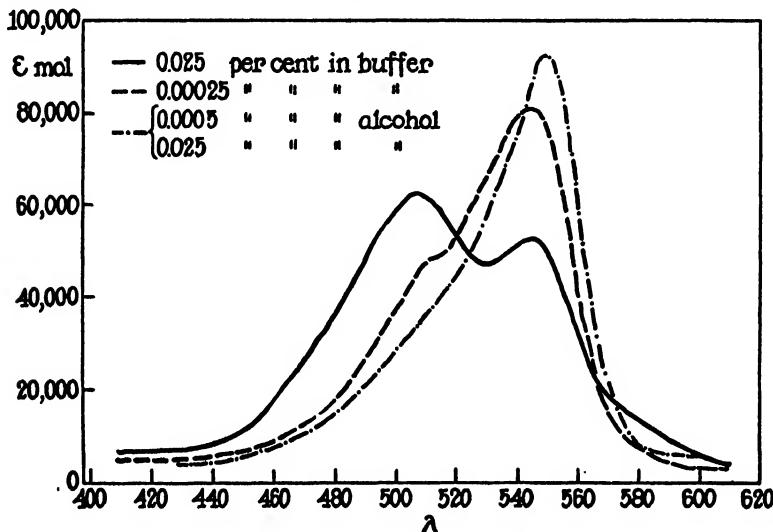
TEXT-FIG. 3. Absorption spectrum of Grübler's pyronin in pH 4.1 acetate buffer and in alcohol.

ever, most experiments were carried out in 0.2 M pH 4.1 acetate buffer as with methyl green. Phenol and glycerin, as prescribed in Unna's mixture, had no influence on the height or shape of the absorption spectrum. In dilute aqueous solutions, the absorption spectrum shows a slight irregularity at 510 mμ and a maximum at 550 mμ. In concentrated solutions, the slight hump at 510 mμ emerges as a definite maximum (Text-fig. 3). Beer's law is followed only in very dilute solutions. In alcohol a smooth curve with maximum at 550 mμ, uninfluenced by concentration, and with a molar extinction coefficient of 91,000, as compared to 79,000 in dilute aqueous solution, is obtained for pyronin Y. Alcoholic solutions obey Beer's law over a wide range of concentrations (Text-fig. 3). In concentrated HCl, a yellow-brown dye with a maximum at 490 mμ and ϵ_{490} = 21,100 results (Text-fig. 4). This follows Beer's law over a wide range also (0.0025 per cent to 0.00025 per cent were compared). The dye content of the various dye preparations was determined by nitrogen determinations on aqueous solutions made up to 0.25 per cent by weight and by comparison of the absorption at

550 mμ of alcoholic solutions with an alcoholic solution of the corresponding per-chlorate (prepared as for methyl green perchlorate from 0.25 per cent solution of pyronin by weight). The several pyronins used and their dye contents were:

Grübler pyronin, 10 per cent of theoretical nitrogen, 12 per cent of theoretical absorption (assumed to be pyronin B).

A. H. Metz pyronin, 13 per cent of theoretical nitrogen, 12 per cent of theoretical absorption (assumed to be pyronin B).

Pyronin Y, National Aniline Corp., certification No. NP 11, "38 per cent dye content," 24.5 per cent of theoretical absorption, 48.5 per cent of theoretical nitrogen (possibly contains contaminating nitrogenous salts).
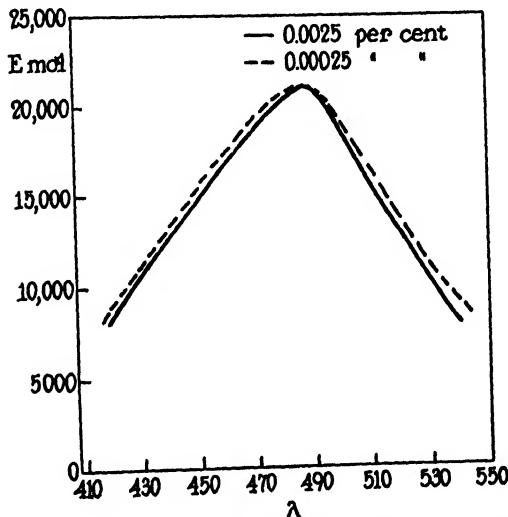
TEXT-FIG. 4. Absorption spectrum of Grübler's pyronin in concentrated hydrochloric acid.

Pyronin B, Coleman and Bell, certification No. CP6, "41 per cent dye content," 53 per cent of theoretical absorption (ϵ_{550} = 104,700 in alcohol as determined for the perchlorate).

3. Ethyl green, C.I. No. 685, National Aniline Corp., certification No. NG-3, dye content 61.9 per cent by absorption, 56.2 per cent by nitrogen content (after CHCl₃ extraction of the solution). This dye was indistinguishable from methyl green in its properties.

4. Malachite green, C.I. No. 657, National Aniline Corp., certification No. NMg 13. This dye gives a slight β hump in concentrated solutions, but insufficient to cause significant deviation from Beer's law. (Cf. Text-figs. 7A and 7B.)

5. Crystal violet, National Aniline Corp.

6. Victoria Blue, National Aniline Corp.

7. Phenosafranin, C.I. No. 840, National Aniline Corp. lot No. 6016. Whereas, unlike pyronin this dye reveals no β hump in its absorption spectrum, alcoholic solu-

tions and aqueous solutions containing DNA reveal maxima at longer wave lengths than simple aqueous solutions (Text-fig. 5). High concentrations in aqueous solutions result in a shift of the maximum to shorter wave lengths (Text-fig. 6).

8. Bismarck brown Y, C. I. No. 331, National Aniline Corp.

Staining with the Unna-Pappenheim Mixture

A simple qualitative test of the relationship between states of polymerization and selective staining by methyl green–pyronin was performed by staining
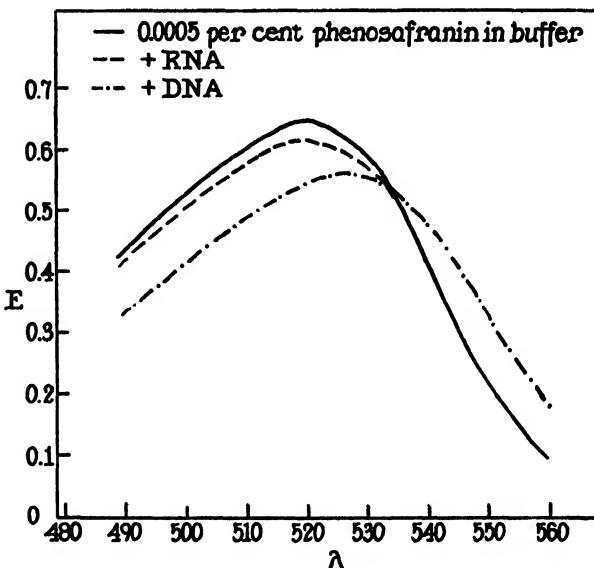
TEXT-FIG. 5. (*a*) Absorption spectrum of 0.0005 per cent phenosafranin in pH 4.1 acetate buffer. (*b*) Same with 4 mg. per cent RNA (P = 0.328 mg. per 100 cc.). (*c*) Same with 4 mg. per cent DNA (P = 0.32 mg./100 cc.).

nucleic acids and nucleoproteins deposited on glass slides. Measured drops of the solutions investigated were pipetted onto albumin-coated glass slides, allowed to dry at 45°C., stained for 30 minutes in the methyl green–pyronin mixture of Unna, rinsed in water, differentiated in 95 per cent alcohol, dehydrated in 100 per cent alcohol and xylene, and mounted in clarite. The solutions used were: (*a*) 0.010 cc. DNH (P = 0.27 mg./cc.), (*b*) 0.025 cc. DNA (P = 0.085 mg./cc.), (*c*) 0.042 cc. depolymerized DNA (P = 0.049), mg./cc.),(*d*) 0.015 cc. RNA (P = 0.152 mg./cc.), (*e*) 0.025 cc. RNP (P = 0.10 mg./cc.) (*f*) 0.2 cc.tobacco mosaic virus (P = 0.014 mg./cc.). After rinsing in water, all the spots appeared purplish brown, but after a few minutes in alcohol, differential staining became apparent. The DNH and DNA spots were bright green, while the depolymerized DNA, RNA, and RNP spots were pink (Fig. 1).

Staining with Dyes Separately

For quantitative and semiquantitative study of the degree of selectivity, it is necessary to work with each dye separately rather than with mixtures. That this was feasible, and that the peculiar selectivity was not a property of the mixture only, could be demonstrated by staining tissue sections with aqueous solution of each dye alone. Cells stained in 0.25 per cent (by weight of
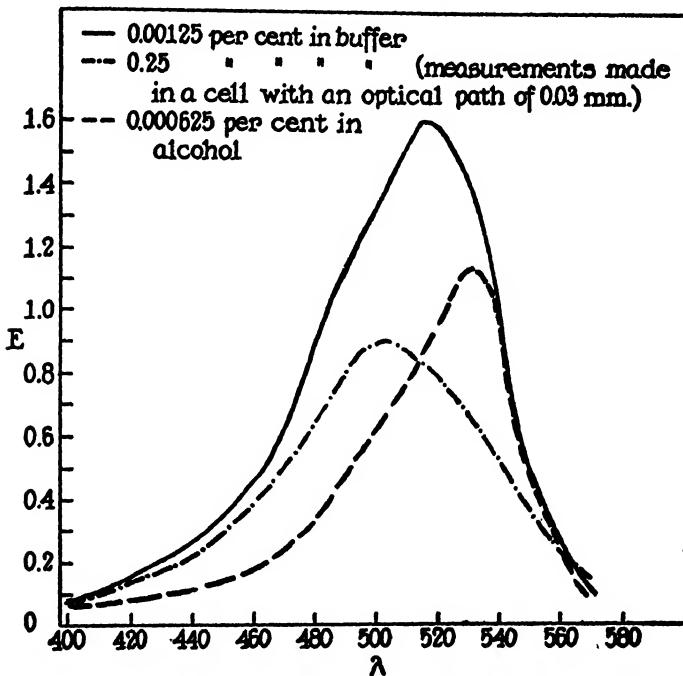
TEXT-FIG. 6. Absorption spectrum of phenosafranin in pH 4.1 acetate buffer and in alcohol.

commercial dye) aqueous methyl green (chloroform-extracted) display green chromatin, while nucleoli and cytoplasm remain unstained. On the other hand, cells stained in 0.25 per cent aqueous pyronin reveal pink cytoplasm and nucleoli, and only very pale pink chromatin (which is consistent with the ribonucleic acid content of chromosomes (3)).

1. Methyl Green Alone.—The addition of DNA to dilute solutions of methyl green results in an increase in the absorption at the maximum, which is shifted from 635 to 645 mμ. It was observed that this change, although apparent at once to the eye as a change in color from blue-green to emerald green, progressed slightly at room temperature for 1 to 2 hours (about 15 per cent increase in E_{645} (optical density at 645 mμ) from zero time) and was stationary thereafter.

For this reason, in all precipitation and dialysis experiments, the mixtures were allowed to stand at room temperature in the dark for at least 2 hours before the precipitant was added or the dialysis begun. This color change was not observed with RNA and depolymerized DNA in the concentrations used in our experiments (0.1 to 0.3 mg. nucleic acid phosphorus in 5 cc. of 0.0025 to 0.005 per cent methyl green). However, ten to twenty times this amount of RNA shifted the absorption maximum to 640 mμ without increasing the extinction coefficient. On precipitation of the nucleic acid with lanthanum acetate, the dye which remains in the supernate is found to have the color and absorption spectrum of pure dye solutions.

A simple qualitative demonstration of the effect of polymerization is based upon this color change in the presence of DNA. To 0.5 cc. 0.028 per cent methyl green (corrected dye content) in 0.2 M pH 4.1 acetate buffer, were added 3.5 cc. buffer and 1 cc. 2 mg./cc. DNA (P = 0.158 mg.). Such mixtures, with controls consisting of 0.5 cc. 0.028 per cent methyl green and 4.5 cc. buffer, were incubated at 0°C., 22°C., 40°C., 60°C., and 100°C. Within 10 minutes, the DNA mixtures kept at 60°C. and 100°C. had changed from emerald green back to the blue-green of the controls, while the mixtures kept at the lower temperatures remained emerald green. Upon the addition of 0.5 cc. 15 per cent lanthanum acetate the solutions maintained for 2 hours at 0°C., 22°C., and 40°C. gave green fibrous precipitates, while those kept at 60°C and 100°C. gave almost colorless granular precipitates. This change in the character of the precipitates indicates that depolymerization of the DNA had occurred at the higher temperatures, with the consequent loss in affinity for methyl green. Reducing the temperature after exposure to 60–100°C. did not reverse the color change in the solutions nor the character of the LaAc$_3$ precipitates.

This observation appeared inconsistent with the reported lack of influence of hydrolysis, as performed in the Feulgen reaction, on methyl green staining (7). We therefore performed the following quantitative experiments.

(a) To 1 cc. 4 mg./cc. DNA (P = 0.320 mg./cc.), 0.5 cc. 3 N HCl was added so as to make a 1 N HCl solution as used in Feulgen hydrolysis; (b) to 1 cc. 4 mg./cc. DNA, 0.5 cc. 0.2 M pH 4.1 acetate buffer was added. These tubes were heated for 12 minutes in a 60°C. water bath and cooled. To (a), 0.4 cc. 5 N NaOH was added to approximately neutralize the solution, 1.9 cc. buffer to bring the pH to 4.1, 1.0 cc. 0.128 per cent methyl green. To (b), 2.3 cc. buffer + 1 cc. 0.128 per cent methyl green was added. After 1 hour at room temperature, 0.2 cc. 1.5 per cent LaAc$_3$ was added to each. Controls consisted of (c) a mixture prepared as in (b) but not heated and (d) a mixture consisting of 1 cc. H$_2$O + 2.8 cc. buffer + 1 cc. methyl green + 0.2 cc. LaAc$_3$. (a) and (b) each gave granular precipitates while the unheated DNA mixture (c) gave a fibrous precipitate. The precipitates were removed by centrifugation, the supernates diluted 1:100 with buffer, and their absorptions compared at 635 mμ. Aliquots of the supernates were used for phosphorus determinations and found to contain none. The absorptions at 635 mμ are given in Table I.

Thus heating DNA at 60°C. for 12 minutes results in considerable loss of staining with methyl green.

A similar experiment was performed with isolated calf liver nuclei (8):

(a) 1 cc. calf liver nuclei in 0.2 per cent citric acid (P_{DNA} = 0.228 mg./cc.) + 0.5 cc. 3 N HCl, heated at 60°C. for 12 minutes, cooled, + 0.4 cc. 5 N NaOH (to approximately neutralize), + 2.1 cc. 0.2 M pH 4.1 buffer (to adjust pH to 4.1) + 1 cc. 0.128 per cent methyl green. (b) 1 cc. nuclei + 3.0 cc. buffer, heated at 60°C. for 12 minutes, cooled, + 1 cc. methyl green. (c) 1 cc. nuclei + 3.0 cc. buffer, let stand at room temperature 12 minutes, + 1 cc. methyl green. (d) 1 cc. nuclei + 1 cc. 0.2 N HCl, let stand 12

TABLE I

Mixture	E_{635}	μM dye in supernate $\dfrac{E_{635} \times 100 \times 10^6 \times 5}{74{,}400 \times 10^3}$	μM dye in precipitate	μM dye/μM P_{DNA}
(a)	0.314	2.11	0.35	0.034
(b)	0.286	1.92	0.54	0.052
(c)	0.239	1.61	0.85	0.083
(d)	0.366	2.46	0	

TABLE II

Mixture	E_{635}	μM dye in supernate	μM dye in precipitate	μM dye/μM P_{DNA}
(a)	0.341	2.29	0.17	0.0231
(b)	0.314	2.11	0.35	0.0476
(c)	0.315	2.12	0.34	0.0463
(d)	0.256	1.72	0.74	0.10
(e)	0.366	2.46	0	

minutes, at room temperature, + 2 cc. buffer + 1 cc. methyl green. (e) 4.0 cc. buffer + 1 cc. methyl green.

After 2 hours, the nuclei were centrifuged off, the supernates diluted 1:100 with buffer, and the extinctions at 635 mμ were compared (Table II).

Thus, heating nuclei in 1 N HCl caused considerable loss of staining with methyl green. Whereas the heating of DNA to 60°C., even in a pH 4.1 buffer caused loss of staining with methyl green, nuclei were not affected by such treatment. The greater staining after treatment with cold dilute HCl is attributed to the displacement of competing histone (cf. similar competition between histone and crystal violet (9)).

Finally, nuclei smeared on glass slides and hydrolyzed for 12 minutes at 60°C. in 1 N HCl, then stained in buffered 0.128 per cent methyl green, failed to take any visible stain, whereas smears placed in 0.1 N HCl for 12 minutes at room temperature stained readily.

We were thus unable to confirm Di Stefano's results (7) and are of the opinion that acid hydrolysis for 12 minutes partially depolymerizes the DNA so as to interfere with methyl green staining. It may be that Di Stefano's findings resulted from the use of unpurified commercial samples of methyl green (personal communication), which contain considerable amounts of crystal violet. Since crystal violet staining is not dependent upon the state of polymerization of the nucleic acids, the loss of methyl green staining may have been obscured.

The effect of the state of polymerization on staining with methyl green was studied also by comparing the amounts of stain bound by nucleic acids precipitated from mixtures of the stain and nucleic acid solutions.

To 5 cc. of a 0.128 per cent solution of methyl green in acetate buffer, solutions of DNA, depolymerized DNA, or RNA containing similar amounts of phosphorus (P = 0.20 to 0.24 mg.), were added and made up to 10 cc. with buffer. The nucleic

TABLE III

	P in precipitate	μM P in precipitate	E_{440} of HCl solution calculated for 10 cc.	μM dye	μM dye/μM P
	mg.				
DNA......................	0.16	5.17	0.561	0.628	0.12
Depolymerized DNA..........	0.12	3.87	0.082	0.092	0.02
RNA......................	0.19	6.13	0.247	0.277	0.045

acid–stain complex was then precipitated with an equal volume of alcohol. The precipitate was washed several times with alcohol, until the washes were colorless. The polymerized DNA precipitate was now a deep green, whereas the depolymerized DNA and RNA were practically colorless. The washed precipitates were dried at room temperature, and dissolved in a known volume of concentrated HCl (4 cc. for RNA and depolymerized DNA, 10 cc. for DNA). The optical density of the brown HCl solution was measured against a concentrated HCl blank at 440 mμ. The phosphorus content of an aliquot of the HCl solution was determined. The results are given in Table III.

As will be noted, the polymerized DNA stains several times as intensely as the depolymerized DNA and RNA. This experiment is to be regarded only as a semiquantitative demonstration of the hypothesis, however, since unstained solutions of nucleic acids dissolved in concentrated HCl in the above concentrations gave E_{440} of 0.032 to 0.057. The effect of this correction on the μM dye/μM P_{DNA} would be to reduce it by less than 10 per cent, but in the case of depolymerized DNA and RNA, significant corrections result. As will be seen in the subsequent paper (10), the stoichiometry for DNA-methyl green found here is reproduced by other methods, whereas the intensity of RNA and depolymerized DNA staining indicated by these results is too great.

The effect of the degree of polymerization of the nucleic acids on staining with methyl green was also investigated by precipitating the stained compound from solution with lanthanum acetate. The amount of dye precipitated was determined by the loss of light absorption in the supernate as compared with a control (containing dye without nucleic acid) and the nucleic acid precipitated was determined by difference (nucleic acid phosphorus added minus phosphorus found in supernate). Since, as will be seen in the subsequent paper on the stoichiometry of the DNA-methyl green reaction (10), lanthanum competes with the dye for the nucleic acid, the results are only of relative value.

TABLE IV

	μM P in precipitate	E_{635} supernate	μM dye in 5 cc. supernate	μM dye in precipitate	μM dye/μM P in precipitate
DNA......................	5.0	0.530	0.356	0.199	0.04
Depolymerized DNA..........	5.68	0.810	0.545	0.010	0.0018
RNA......................	5.3	0.820	0.552	0.003	0.0006
Control....................	0	0.825	0.555	0	

TABLE V

	μM P in precipitate	E_{635} supernate	μM dye in 5 cc. supernate	μM dye in precipitate	μM dye/μM P in precipitate
DNA......................	5.1	0.121	0.081	0.223	0.044
Depolymerized DNA..........	5.62	0.445	0.295	0.009	0.0016
Control....................	0	0.452	0.304	0	

To 1 cc. 0.025 per cent dye in buffer + 2.5 cc. buffer, was added 1 cc. of DNA (P = 0.155 mg./cc.), depolymerized DNA (P = 0.176 mg./cc.), or RNA (P = 0.164 mg./cc.). After 2 hours, 0.5 cc. 15 per cent LaAc₃ was added, the precipitates removed by centrifugation, and the absorptions of the supernates at 635 mμ compared after diluting 1:10. The control contained 1 cc. H₂O instead of nucleic acid solution. The results are tabulated in Table IV.

The same experiment, using one-half the dye concentration and 0.5 cc. 0.6 per cent LaAc₃ as the precipitant instead of 0.5 cc. 15 per cent LaAc₃, gave the results tabulated in Table V.

Thus we note that depolymerization of the DNA has resulted in a 25-fold decrease in staining with methyl green.

As an indication of the specificity of staining by methyl green, and of the rôle of polymerization of carbohydrates in staining with this dye, 1 cc. of a solution of Pneumococcus Type III polysaccharide[2] (2 mg./cc.) was mixed

[2] The bacterial polysaccharides were provided by Dr. Goebel of The Rockefeller Institute for Medical Research.

with 4 cc. of buffered 0.003 per cent methyl green. No change in the absorption spectrum of the solution, such as has been described above with DNA, occurred. To 5.5 cc. of this mixture, 0.5 cc. 15 per cent lanthanum acetate was added. The flocculent precipitate which resulted was practically colorless, and the supernate revealed no loss of dye as measured by its absorption at 635 mμ. The very faint blue-green color in the precipitate is rapidly removed with alcohol. On the other hand, crystal violet applied in place of methyl green yields a deep purple precipitate which retains most of its color despite repeated washing with alcohol (crystal violet stains both RNA and DNA without selectivity).

Smears of capsulated Friedländer's bacillus did not stain at all with methyl green. When stained in the Pappenheim mixture (methyl green–pyronin), the organisms from young broth cultures appeared as pink outlines containing red polar bodies. A similar picture of a dark outline with two dense polar bodies is seen in the phase contrast microscope.

2. Pyronin Alone.—It was found that in sufficiently high concentration, pyronin caused the precipitation of nucleic acids and nucleoproteins from solution. It was thus possible to study the effect of the state of polymerization of nucleic acids on pyronin staining by precipitation with pyronin alone or, when dilute pyronin solutions were used, with the aid of alcohol or LaAc$_3$.

To 5 cc. (a) 1 mg./cc. DNA (P = 0.08 mg./cc.), (b) depolymerized DNA (P = 0.05 mg./cc.), (c) 1 mg./cc. RNA (P = 0.09 mg./cc.), (d) RNP (P = 0.1 mg./cc.), and (e) DNH (P = 0.11 mg./cc.), 5 cc. of 0.0625 per cent aqueous solution of pyronin Y (National Aniline) was added. In each case a precipitate resulted (fibrous in the case of DNA and DNH, granular in the others). Upon removal of the precipitates by centrifugation (the fibrous precipitates could usually be removed with a glass rod without centrifugation), the supernates were analyzed (a) spectrophotometrically for dye by comparison of the absorption at 550 mμ to a control dye solution and (b) by the method of Allen (11) for phosphorus content. The results are given in Table VI.

A similar experiment was performed with Grübler's pyronin as follows:—

Control: 2 cc. 0.025 per cent pyronin in pH 4.1 acetate buffer + 3 cc. H$_2$O.
RNA : 2 cc. 0.025 per cent pyronin + 2 cc. H$_2$O + 1 cc. 20 mg./cc. RNA (P = 1.58 mg./cc.).[3]
DNA : 2 cc. 0.025 per cent pyronin + 2 cc. H$_2$O + 1 cc. 2 mg./cc. DNA (P = 0.160 mg./cc.).

RNA yielded a granular precipitate and DNA a swollen fibrous precipitate. The precipitates were removed by centrifugation and the supernates analyzed for dye and phosphorus content as above. The results are presented in Table VII.

We can see from the two following tables that depolymerized DNA, RNA, and RNP stain similarly, while DNA and DNH reveal less affinity for pyronin.

[3] The large amount of RNA is required to obtain a significant precipitate.

With pyronin, therefore, as with methyl green, the state of polymerization of nucleic acid appears to influence the degree of staining.

Samples of nucleic acids and nucleoproteins containing 0.07 to 0.15 mg. P were precipitated from solution by the addition of alcohol and a little salt. These precipitates were stained with 0.025 per cent Grübler's pyronin. Such precipitates, when washed with alcohol, continued to lose stain for many days

TABLE VI

	DNA	Depoly-merized DNA	RNA	RNP	DNH	Control
P content of precipitate, *mg*	0.4	0.2	0.3	0.44	0.53	0
μM	12.9	6.5	9.7	14.2	17.1	
Dye content of supernate, expressed as E_{550} diluted 1:100	0.671	0.614	0.609	0.404	0.761	0.875
Dye content of supernate, μM*	8.5	7.77	7.7	5.11	9.6	10.9
Dye content of precipitate, μM	2.4	3.1	3.2	5.8	1.3	0
Dye/P, molar	0.19	0.48	0.33	0.41	0.076	

$$* = \frac{E_{550} \times 1000}{79}.$$

TABLE VII

	Control	RNA	DNA
P content of original mixture, *mg*	0	1.58	0.16
P content of supernate, *mg*	0	1.54	0.034
P content of precipitate, *mg*	0	0.04	0.13
μM		1.29	4.1
E_{635} of supernate, diluted 1:30	0.895	0.359	0.094
Pyronin in supernate, μM	1.70	0.682	0.179
Pyronin in precipitate, μM	0	1.02	1.52
Dye/P, molar		0.79	0.37

but quickly reached an end point with acid alcohol. Unlike pyronin Y, which is completely removed from nucleic acid precipitates by acid alcohol, a small amount of Grübler's pyronin remains attached. Such precipitates, washed with acid alcohol, were completely dissolved in a small volume of concentrated HCl and compared spectrophotometrically. The results were reproducible within 15 per cent (as contrasted with the variable results obtained with washed pyronin Y precipitates). The results of four experiments are averaged in Table VIII.

In another effort to compare the staining of nucleic acid solutions with pyronin, dilute solutions of pyronin and nucleic acids were mixed and the nucleic acid–stain complex precipitated with lanthanum acetate, as was done with

methyl green. Experiments were set up in acetate buffer so that the final concentration of dye was 0.0025 per cent (except pyronin B, where 0.004 per cent

TABLE VIII

	DNA	Depolymerized DNA	RNA	RNP	DNH
Dye/P, molar..............	0.0035	0.02	0.02	0.02	0.0023

TABLE IX

	Control	RNA	DNA	Depolymerized DNA
Pyronin Y				
Nucleic acid P in mixture, *mg*............		0.162	0.155	0.174
in supernate, *mg*..........		0.086	0	0.125
in precipitate, *mg*........		0.076	0.155	0.049
P in precipitate, μM.....................		2.44	5.0	1.61
E_{550} supernate, diluted 1:10..............	0.608	0.419	0.482	0.515
Pyronin Y in precipitate, μM (ϵ_{550} = 79,000).		0.138	0.087	0.062
Dye/P molar...........................		0.057	0.017	0.039
Grübler's pyronin				
Nucleic acid P in mixture, *mg*............	0	0.131	0.128	
in supernate, *mg*..........		0.036	0.003	
in precipitate, *mg*........		0.095	0.125	
P in precipitate, μM.....................		3.06	4.03	
E_{547} supernate, diluted 1:10..............	0.619	0.281	0.380	
Pyronin Grübler in supernate, μM (ϵ_{547} = 79,000)...............................	0.353	0.160	0.217	
Pyronin Grübler in precipitate, μM........		0.193	0.136	
Dye/P, molar....................,.......		0.063	0.033	
Pyronin B				
Nucleic acid P in mixture, *mg*............		0.162	0.155	
in supernate, *mg*..........		0.047	0	
in precipitate, *mg*........		0.115	0.155	
P in precipitate, μM.....................		3.7	5.0	
E_{550} supernate diluted 1:30..............	0.432	0.344	0.390	
Pyronin B in precipitate, μM (ϵ_{550} = 87,900).		0.15	0.072	
Dye/P, molar...........................		0.04	0.014	

was used) and of nucleic acid phosphorus about 0.0032 per cent. To 4.5 cc. of these mixtures, 0.5 cc. 1.5 per cent lanthanum acetate was added, the precipitates were removed, and the supernates analyzed for dye content and phosphorus. The results are given in Table IX.

These experiments thus demonstrate a two- to threefold greater staining by pyronin of low polymers of nucleic acid than of high polymers.

It should be noted that in the mixtures described in these experiments, using dilute dye solutions, DNA changes the color of the solution from orange-red to rose-red with a shift in the absorption maximum from 550 mμ to 565 mμ. This change was much less marked with similar concentrations of RNA or depolymerized DNA, but did occur when the concentration of RNA was increased tenfold.

Staining with Related Dyes

The staining of nucleic acids by several other dyes was investigated in the hope that some light might be shed upon the structural determinants of dye selectivity. The experiments were set up as follows:—

Control: 0.5 cc. 0.05 per cent dye (by weight of commercial powder) in 0.2 M pH 4.1 acetate buffer + 4.3 cc. buffer + 0.2 cc. 1.5 per cent LaAc₃.

RNA: 0.5 cc. dye + 3.3 cc. buffer + 1 cc. 2 mg./cc. RNA (P = 0.164 mg.) + 0.2 cc. LaAc₃.

DNA: 0.5 cc. dye + 3.3 cc. buffer + 1 cc. 2 mg./cc. DNA (P = 0.160 mg./cc.) + 0.2 cc. LaAc₃.

Depolymerized DNA: 0.5 cc. dye + 2.3 cc. buffer + 2 cc. depolymerized DNA (P = 0.087 mg./cc.) + 0.2 cc. LaAc₃.

The composition of the precipitates was calculated from the change in the extinction coefficient (at the maximum absorption of the dye) of the supernate upon precipitation of the nucleic acid, and from the residual phosphorus content of the supernate.

The following triphenylmethane dyes were examined in order to compare their selectivity to methyl green:

(a) Ethyl green:
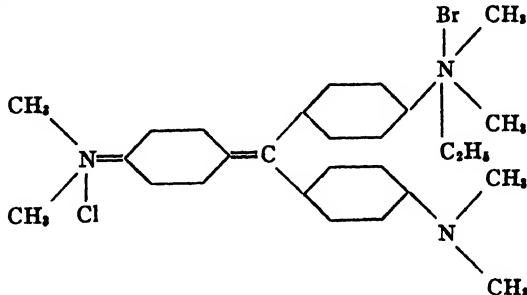
National Aniline Corp., certification No. NG-3, dye content 61.9 per cent by absorption, 65.2 by nitrogen content (after CHCl₃ extraction of solution).

(b) Malachite green:
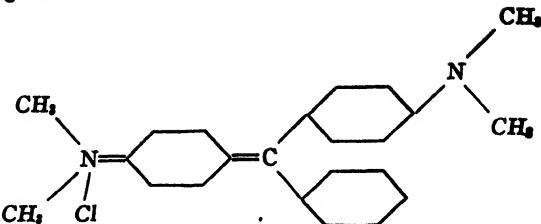
C. I. Number 657, National Aniline Corp., certification No. NMg 13

(c) Crystal violet:
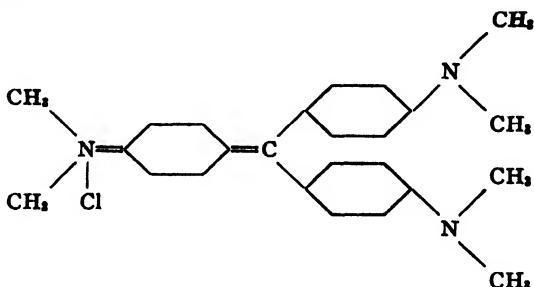
National Aniline Corp., C. I. Number 681.

(d) Victoria blue:
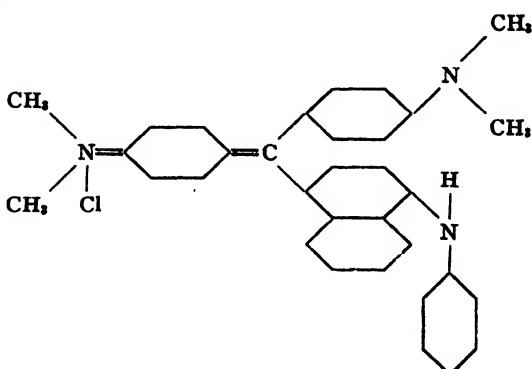
National Aniline Corp., C. I. Number 729.

The results are tabulated (Table X). The values must be compared only for each dye, since no correction was made for dye content except for methyl green and ethyl green (based upon $\epsilon_{635} = 74,400$).

Even then, the values are of relative significance only, since the use of dilute dye solutions and lanthanum is not optimum for stoichiometric study. (See above and following paper (10).)

Ethyl green showed the same color change with DNA as did methyl green, and the same order of selectivity. Malachite green also demonstrated a shift

in the position of the absorption maximum from 620 mμ to 630 mμ with DNA but not with RNA or depolymerized DNA. It also revealed selectivity for the higher polymer, but to a lesser degree than did methyl green. Crystal violet and Victoria blue failed to demonstrate any selectivity.

TABLE X

Dye	Dye/P, Molar		
	RNA	DNA	Depolymerized DNA
(1) Methyl green	0.004	0.05	0.004
(2) Ethyl green	0.007	0.04	0
(3) Malachite green	0.006	0.017	0.007
(4) Victoria blue	0.13	0.1	0.12
(5) Crystal violet	++	++	++

In the same manner we examined phenosafranin,
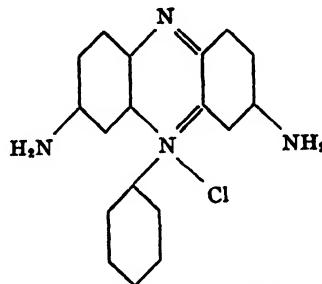
C. I. Number 840, National Aniline Corp., lot Number 6016

and Bismarck brown Y,
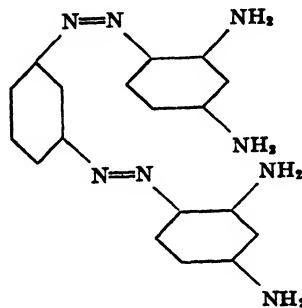
C. I. Number 331, National Aniline Corp., certification No. NN9.

Phenosafranin was selected because it has been recommended as a nuclear stain, and, like methyl green, has been used as a stain for lignin. Bismarck brown was chosen because it has been recommended as a stain for mucin in con-

trast to methyl green (12). Both of these dyes failed to distinguish between the nucleic acids. Bismarck brown Y, even in dilute solutions, caused the precipitation of nucleic acids. It therefore provided an opportunity to compare the reaction with and without lanthanum. It was found that here, as with methyl green (10), lanthanum competed with the dye for the nucleic acid in that the addition of 1.5 per cent lanthanum acetate reduced the amount of stain bound by the nucleic acids by 55 per cent.

DISCUSSION

Whereas, we have observed that staining of DNA and presumably also RNA by methyl green and pyronin is a function of the state of polymerization of the nucleic acid substrates, we can only guess at the mechanism of the selection. The fact that mixtures of methyl green and pyronin are selectively adsorbed by strips of filter paper or permutit columns, suggests that the staining of nucleic acids may be, at least in part, a physical phenomenon, with selective adsorption determined by the physical state of the substrate molecules. However, the constant stoichiometry of methyl green staining of polymerized DNA, and the competition of lanthanum and of histone (10) point to chemical union with the phosphoric groups.

We have seen that triphenylmethane dyes with two methyl-amino groups, such as methyl green, ethyl green, and malachite green are selective for polymerized nucleic acids, while those with three amino groups, such as crystal violet and Victoria blue show no such selectivity. This appears particularly significant when one contrasts methyl green and crystal violet, in which the only difference lies in the methylation of one amino group of crystal violet, thus converting it into the quaternary ammonium ion of methyl green. Crystal violet stains both DNA and RNA with one dye molecule combining with two phosphoric groups (9), while methyl green stains polymerized DNA with one dye molecule per 10 phosphoric groups (10), and practically fails to stain RNA or depolymerized DNA. This suggests that the triphenylmethane dyes are bound to nucleic acid molecules by two amino groups. This requires that the spacing between pairs of phosphoric groups match that between a pair of amino groups in the dye molecule. In the case of crystal violet, three such spacings are possible, while in the methyl green molecule, only one exists. It may be that in highly polymerized DNA, every fifth pair of phosphoric groups presents the appropriate spacing, while in depolymerized DNA and in RNA only rarely does the correct spacing occur. The much less strict requirements of crystal violet permit all pairs of phosphoric groups to participate.

The selectivity of pyronin is even more puzzling. However, it is possible that this molecule combines preferentially with the dibasic phosphoric acid groups. If nucleotides are indeed linked as indicated by Levene's structure

```
                                        O
                                        ‖
        Purine—sugar—O—P—OH
                        \        \
                         O        OH
                          \        O
        Pyrimidine—sugar—O—P—OH
                          |
                          O  O
                          |‖
        Purine—sugar—O—P—OH
                        \
                         O      O
                          \     ‖
        Pyrimidine—sugar—O—P—OH
```
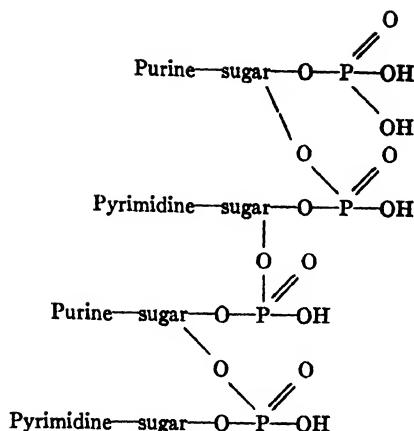
it follows that a high polymer will offer fewer dibasic groups than a low polymer. Thus, a tetranucleotide would contain one dibasic phosphoric acid and a 40-nucleotide would likewise contain only one dibasic group.

Similar considerations may explain our failure to observe staining of highly polymerized bacterial polysaccharides with methyl green. Nevertheless, it is possible that still higher degrees of polymerization would afford steric opportunities for staining with this dye. This is suggested by the observation that lignin, but not cellulose and mucin, is stained by methyl green (13). Whereas these polysaccharides have different chemical compositions, on maceration with strong nitric acid and potassium chlorate, lignin gives the color reactions of cellulose with iodine and sulfuric acid, and with zinc chloroiodide. This treatment very likely causes partial depolymerization of the lignin. Methyl green staining is lost following maceration (13). Basic dyes such as Bismarck brown and crystal violet, which lack the selectivity of methyl green, stain mucin and plant cellulose as well as lignin.

We have observed that dilute solutions of DNA shift the absorption maxima of methyl green, ethyl green, malachite green, phenosafranin, and pyronin to longer wave lengths, while RNA does so only in much more concentrated solutions. We believe that the shift in the absorption spectrum is a manifestation of the orientation of the dye by its attachment to an oriented structure (the fibrous DNA polymer in this case). Such orientation may also be construed as preventing polymerization of the dye, as suggested by Michaelis (14), but then we must assume polymerization, at least to a slight degree for methyl green, malachite green, and phenosafranin, which Michaelis considered always to be monomers. Indeed, such a possibility is suggested by the slight irregularity in the ascending limb of the absorption spectrum found in concentrated solutions of malachite green which disappears on dilution, suggesting a β curve (Text-fig. 7B), on the shift in the absorption spectrum of malachite green when dis-

solved in chloroform (resembling that in DNA) (Text-fig. 7A), and by the shift in the phenosafranin spectrum on dilution of aqueous solutions or in alcohol (resembling that in DNA) (Text-figs. 5 and 6). Since neither methyl green nor phenosafranin ever exhibit a β maximum to indicate the presence of dimers, we must assume that the polymeric forms have maxima so close to that of the monomer as to result in smooth curves (note that mixtures of excesses of methyl green with DNA, which give absorption maxima between 635 mμ and 645 mμ
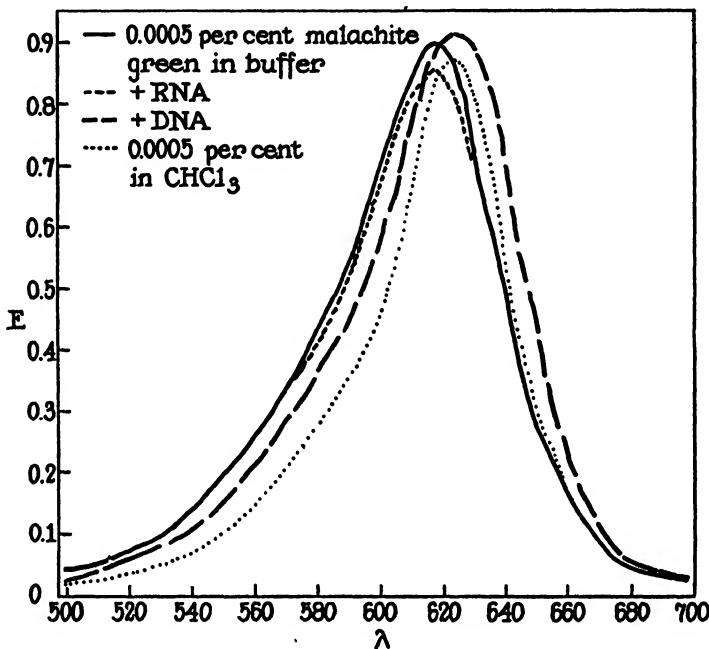
TEXT-FIG. 7A (*a*) Absorption spectrum of malachite green in pH 4.1 acetate buffer. (*b*) Same with 4 mg. per cent RNA (P = 0.328 mg./100 cc.). (*c*) Same with 4 mg. per cent DNA (P = 0.32 mg./100 cc.). (*d*) Malachite green in chloroform.

(indicating a mixture of the uncombined dye and the dye-DNA complex) still give smooth curves (10)). That such orientation of the dye by the nucleic acid may be the factor which determines the shift in the absorption spectrum, is indicated by the failure of dilute RNA solutions to cause the shift, even when the dye is not in excess (particularly with such dyes as phenosafranin and pyronin which stain RNA readily). The smaller RNA fails to provide the backbone for the orientation of neighboring dye molecules. However, when the concentration of RNA is increased manyfold, the shift is noted, suggesting that the "crowding" of the short linear (?) RNA molecules has imposed some orientation upon them. To state the hypothesis in another way, we can think of a dilute solution of methyl green as containing molecules of the dye moving

freely and at random and free to interact with eac other. The addition of suitably polymerized nucleic acid molecules, containing chains of 20 or more nucleotides will bind two or more dye molecules to a single straight chain, thus restricting the motion of the dye molecules and preventing their interaction. Chains of less than 20 nucleotides will bind 0 to one molecule of dye and thus not significantly influence the opportunity for interaction. The shift of the absorption maxima by DNA toward longer wave lengths is that usually seen with dyes which are known to polymerize (14).
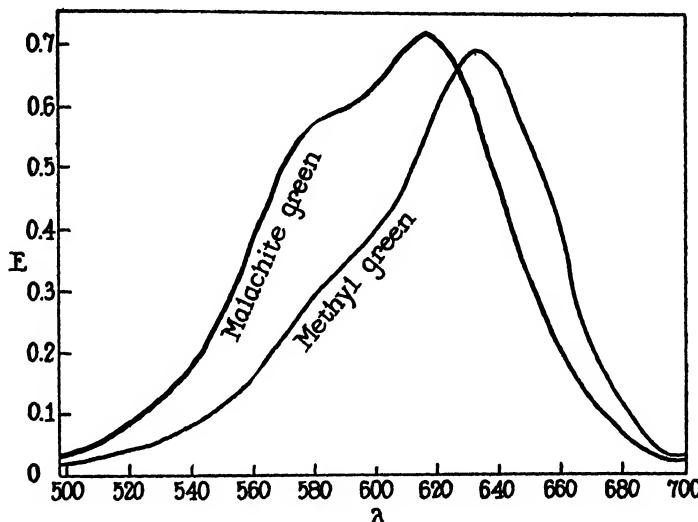
TEXT-FIG. 7B. Absorption spectrum of malachite green and methyl green in pH 4.1 acetate buffer, dye concentration 0.25 per cent, 0.03 mm. thickness.

The effect of polymerization on staining with methyl green–pyronin accounts for the occasional failure to obtain methyl green staining of nuclei in tissue sections. This is most apt to occur after aqueous acid fixation, such as Zenker's, where prolonged contact with the acid permits depolymerization of the DNA. Special care in the handling of tissues to avoid such depolymerization is essential if methyl green–pyronin is to be applied as a histochemical technique.

SUMMARY

1. Methyl green stains selectively highly polymerized desoxyribonucleic acid, and fails to stain, to any significant extent, depolymerized desoxyribonucleic acid and ribonucleic acid.

2. Pyronin stains preferentially low polymers of nucleic acid.

3. Triphenylmethane dyes with two amino groups appear to share the selectivity of methyl green. Those with three amino groups are not selective.

4. A stereochemical hypothesis is offered to account for these observations.

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EXPLANATION OF PLATE 1

FIG. 1. Unna-Pappenheim stain of nucleic acids, showing effect of depolymerization.

Desoxyribonucleohistone

Desoxyribonucleic acid

Depolymerized desoxyri-
bonucleic acid

Fig. 1

(Kurnick: Methyl green-pyronin. I)

METHYL GREEN–PYRONIN

II. Stoichiometry of Reaction with Nucleic Acids

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We have observed (1) that the selective staining of nucleic acids by methyl green and pyronin is a function of the relative states of polymerization of the nucleic acids. The purpose of this paper is to present studies on the stoichiometry of the reactions, in order to elucidate the mechanism of the selectivity and to explore the possibility of quantitative histochemical application.

The studies, as applied to pyronin, met with only very limited success. Unlike methyl green, which appears to form a stable compound with polymerized DNA, pyronin-stained RNA or depolymerized DNA-precipitates lose stain continuously when washed with alcohol or aqueous buffers. The "end point" which is eventually reached, represents the retention of only a few per cent of the original dye bound (*cf.* Table VI and Table VIII of the preceding paper (1)). Consequently, the possibility of non-specifically adsorbed dye to the pyronin-nucleic acid complex which precipitates on the addition of pyronin to nucleic acid solutions (in high concentrations of dye) cannot be excluded, thus vitiating the significance of the stoichiometric data based upon precipitation experiments in which combined dye was determined by difference between that remaining in the supernate and the original concentration. Likewise, washing probably results in considerable dissociation of the complex so that the "end point" is also not reliable for stoichiometry. However, Table VI (preceding paper (1)) based on precipitation experiments, suggests that the low polymers of NA bind approximately one pyronin molecule for each pair of phosphoric acid groups, indicating that both amino groups are functional in the linkage.

Referring again to Table VI (1), a significant difference in staining is noted between DNA and DNH. We doubt that the difference between RNA and RNP is significant, since the preparations are obtained from different sources by unrelated methods. The possibility that the slightly greater staining of RNP is due to the staining of protein by pyronin is rendered unlikely, but by no means excluded, by the fact that heat-coagulated beef serum fails to stain with pyronin when treated in the same manner as the nucleic acid precipitates.

The interference by histone with the staining of the nucleic acid by pyronin was confirmed by staining fibers of desoxyribonucleohistone prepared by precipitating the nucleohistone from 1 M NaCl solution by dilution with water. The fibrous precipitates (P = 0.27 mg. each) were wound about glass rods. One sample was immersed in 0.2 N HCl and then in pH 4.1 acetate buffer, another only in buffer. They were then transferred to 0.025 ger cent Grübler pyronin, washed in alcohol until the alcohol washes were colorless, and finally decolorized in 10 cc. acid alcohol at 60°C. overnight. Practically complete decolorization is achieved (cf. the low values for retained pyronin after acid alcohol washing of DNA and DNH in Table VIII (1)). The acid alcohol extracts were compared spectrophotometrically for dye content. The results indicated a molar dye/P ratio of 0.0117 for the nucleohistone which had been freed of practically all its histone by the acid treatment and 0.0072 for the intact nucleohistone (Table XI).[1]

TABLE XI

	$E_{\text{λ}}$ acid alcohol extract	μM dye in acid alcohol, based on $\epsilon_{\text{λ}}$ = 91,000	μM P	Dye/P, molar
DNA........................	0.925	0.102	8.7	0.0117
DNH........................	0.570	0.0627	8.7	0.0072

This competition of the histone with pyronin, but not of the more complex protein of ribonucleoprotein suggests that both the dye and histone are attached to the nucleic acid by phosphoric acid groups, while the protein of RNP is bound in a different manner.

It should be noted that the problem of the stoichiometry of pyronin staining is further complicated by the dependence of the degree of staining on the dye concentration. Thus, when the pyronin concentration in the stain-nucleic acid mixture was 0.03 per cent, the dye/$P_{\text{depolymerised DNA}}$, molar = 0.48 (Table VI (1)), whereas in the lanthanum precipitation experiments with a dye concentration of 0.0025 per cent, the dye/$P_{\text{depolymerised DNA}}$, molar = 0.04 (Table IX (1)). Even relatively small differences in dye concentration influenced the degree of staining. Thus, the precipitates obtained in the following three experiments were compared:

(a) 5 cc. RNP (P = 0.1 mg./cc.) + 5 cc. 0.0625 per cent pyronin Y, (b) 3 cc. RNP + 2 cc. H₂O + 5 cc. pyronin Y, (c) 5 cc. RNP + 2 cc. H₂O + 3cc. pyronin Y. The resultant precipitates contained (a) 0.48 mg. P and 8.3 mg. dye, correspond-

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[1] The tables in the present paper are numbered consecutively with those in the preceding paper (1).

ing to a molar ratio of dye: P of 0.45, (b) 0.3 mg. P and 6.2 mg. dye, corresponding to dye/P, molar — .54, (c) 0.42 mg. P and 5.7 mg. dye, with a dye/P, molar of 0.35.

Pyronin, therefore, appears to be unpromising as a quantitative histochemical stain, although as a qualitative test for the presence of RNA or depolymerized DNA as distinguished from polymerized DNA, after appropriate washing with alcohol, it appears to be of value. The specificity is enhanced by the use of ribonuclease (2).
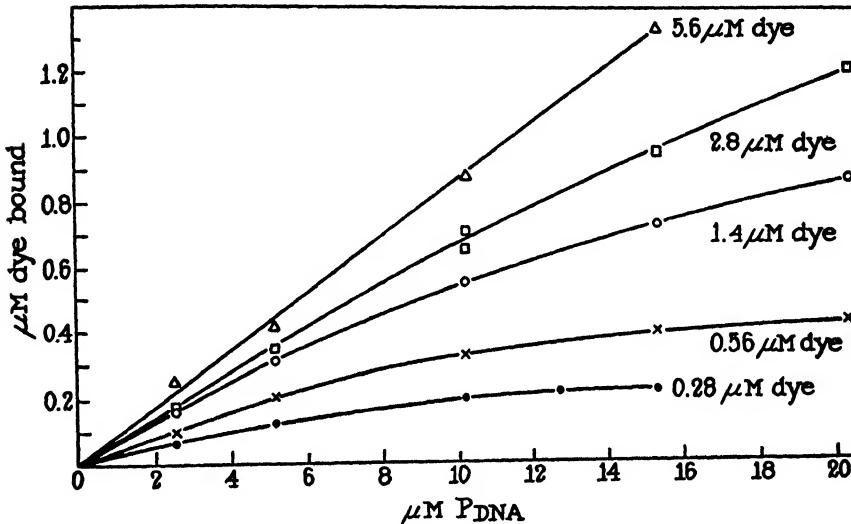
TEXT-FIG. 1. Competition of methyl green and lanthanum acetate for DNA. Final volume 5 cc. in pH 4.1 acetate buffer. Content of dye (on curve) and DNA (on abscissa) varied as indicated. Ordinates indicate amount of dye bound in precipitate (DNA content of precipitate same as on abscissa). Lanthanum acetate final concentration 1.5 per cent.

Much more satisfactory results were obtained in the investigation of the stoichiometry of methyl green. From Table II of the preceding paper (1), we note that after treatment at room temperature with dilute HCl, calf thymus nuclei combine with methyl green in the ratio of 0.1 molecule of dye per phosphorus. The same value is found for the composition of the DNA-methyl green compound precipitated with alcohol (Table III (1)). Lower values were obtained in the experiments in which LaAc₃ was used as the precipitant (Tables I, IV, V (1)). This suggested that lanthanum competed with methyl green for the phosphoric acid groups of DNA. The experiment was set up so that the final volume of 4.5 cc. contained from 0.28 μM to 5.6 μM methyl green and 2.5 μM to 20.4 μM P_DNA in pH 4.1 acetate buffer. After these mixtures had been allowed to stand for two hours, 0.5 cc. 15 per cent LaAc₃ was added to each,

the fibrous precipitate removed with a glass rod, and the extinction coefficients of the supernates at 635 mμ determined. By comparison with controls containing no nucleic acid, the methyl green removed could be calculated. The precipitation of the DNA was quantitative under these conditions. As seen in Text-fig. 1, as the effective excess of dye is increased, the competition of the LaAc$_3$ becomes less effective, until finally it becomes insignificant and a straight line, indicating constant composition of the precipitated compound, regardless of the relative concentrations of the components in the original solution, results. This compound corresponds to a molar dye/P$_{DNA}$ ratio of 0.09, which is consistent with the value of 0.1 obtained in the staining of nuclei and on alcohol precipitation of DNA-methyl green. RNA and depolymerized DNA, under the conditions which give this straight line curve for DNA, combine with only 0.004 molecule of dye per phosphorus.

TABLE XII

Methyl Green/P$_{DNA}$, Molar

Stain in bath	P in mixture	P in super-nate	P in precipi-tate	P in precipi-tate	E_{635} mμ of supernates					Dye/P$_{DNA}$, molar
					Control a diluted 1:20	a diluted 1:20	Control b diluted 1:100	b diluted 1:100	Dye in precipi-tate (e_{635} = 74,400)	
	μM	*mg.*	*mg.*	*mg.*	μM					μM	
(a) 1.41	0.31	0.013	0.297	9.6	0.970	0.438			0.72	0.07
(b) 5.64	0.31	0	0.31	10.0			0.768	0.650	0.92	0.09

The competition with lanthanum could also be demonstrated by washing a sample of stained, precipitated DNA with alcohol until no further dye could be removed. This is accomplished quickly, since methyl green required no "differentiation"—it is only necessary to wash away the stain solution in contact with the sediment with alcohol. Washing with 0.2 M acetate buffer will now remove no dye from the green precipitate. Fifteen per cent LaAc$_3$, will, however, remove a considerable amount of stain as judged by the intensity of color in the wash fluid. Thirty per cent NaCl is practically ineffective in this regard (cf. reference 3). Similarly, staining with methyl green in the presence of 1 M NaCl reduces the amount of dye combined by only 10 per cent.

When the LaAc$_3$ concentration was reduced to the minimum which would precipitate the polymerized DNA quantitatively (0.06 per cent), even with relatively small excess of dye, the molar dye/P$_{DNA}$ ratio was 0.07 to 0.09 (Table XII). These experiments were set up as follows:—

(a) 0.5 cc. 0.128 per cent methyl green + 2.3 cc. buffer + 2 cc. 2 mg./cc. DNA (P = 0.155 mg./cc.) + 0.2 cc. 1.5 per cent LaAc$_3$ (corresponding to 1.41 μM dye and 10 μM P$_{DNA}$).

(b) 2.0 cc. 0.128 per cent methyl green + 0.8 cc. buffer + 2 cc. DNA + 0.2 cc. 1.5 per cent LaAc₃ (corresponding to 5.64 μM dye + 10 μM P_{DNA}).

The controls were made up with water in place of DNA solution.

That histone also competes with methyl green staining is suggested by the observation (Table II of preceding paper (1)) that nuclei stain with only one-half as much methyl green before HCl treatment as after.

Whereas when a solution of methyl green in acetate buffer was dialyzed against acetate buffer, all the dye escaped from the cellophane sac, when DNA was first added to the dye, a considerable amount of dye failed to dialyze. While no absolutely steady state was reached, after prolonged dialysis with frequent

TABLE XIII

Time of dialysis	E_{644} dialyzed solution, diluted 1:20		μM dye per 10 cc. $\epsilon_{644} = 10^5$	μM P per 10 cc.	Dye/P, molar
	Control	Experimental			
hrs.					
26	0.221	0.531		10.3	
29	0.029	0.483	0.986	9.5	0.103
50		0.510	1.02		
74		0.488	0.976		
79		0.459	0.918	10.1	0.091
25	0.221	0.571		10.3	
27	0.062	0.534	1.068		0.103
32	0.015	0.520	1.04		
54		0.477	0.954		0.093
151		0.440	0.88		0.087
343		0.407	0.814	10.1	0.081

or constant changing of the dialysate, the rate of loss of dye from the mixture of DNA and dye became quite slow. This approximate end point corresponded to a composition of the DNA-methyl green complex retained in solution in the cellophane sac of 0.08 to 0.10 mols dye per P.

The experiment was performed by mixing 10 cc. 2 mg./cc. DNA (P = 0.160 mg./cc.) + 20 cc. 0.05 M acetate buffer (pH 4.1) + 20 cc. 0.128 per cent methyl green in 0.2 M acetate buffer. After standing overnight in the dark, the mixture was dialyzed in a cellophane bag against 0.05 M acetate buffer in a rocking, continuous flow dialyzer. Samples of the contents of the bag were withdrawn at intervals. The control contained water in place of DNA solution. The results are presented in Table XIII.

It was not possible to perform comparable experiments with RNA and depolymerized DNA because of the ease with which these nucleic acids passed through the membrane.

Mixtures of DNA and methyl green were prepared so that the ratio dye/P_{DNA} varied over the range 0.05 to 0.225, while the dye concentration was kept constant at 0.005 per cent. Four cc. of each mixture was dialyzed against 10 cc. of acetate buffer at 0°C. in the dark (without changing the dialyzing bath). At infrequent intervals varying from 6 hours to 1 month, the extinction coefficients of the dialysates were measured at 635 mμ. When the extinction values at a given time were plotted against the molar dye/P ratio of the original mixture, hyperboles resulted. Extrapola-
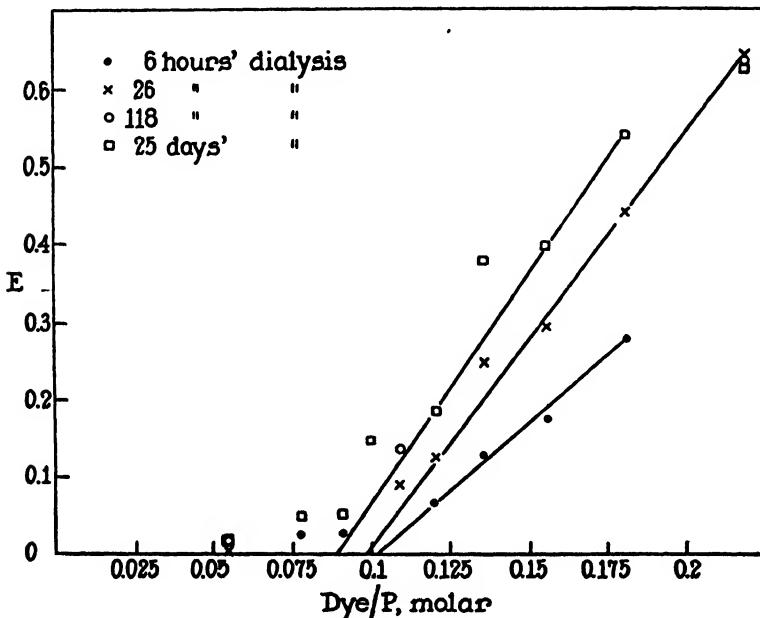
TEXT-FIG. 2. Extinction coefficients of dialyzing bath plotted against methyl green/P_{DNA} of original mixture after dialysis for fixed periods of time (see text for description of experiment). Note that amount of dye which dialyzes out is directly proportional to excess of dye (*i.e.*, when dye/P, molar exceeds 0.1).

tion of the "straight" portion of the curves to zero dye dialysis gave a dye/P of 0.1 (Text-fig. 2). It was found that under these conditions of dialysis, in which the volume of the dialyzing bath was small and the bath was not changed during the course of the experiment, a state of equilibrium was reached in 6 to 15 days. At the end of a month, when it was apparent that no further dialysis of stain was occurring (contrast the situation in which maintaining the concentration of dye in the bath at a minimum by continuous flow dialysis, favored continuous slow dissociation of the DNA-stain complex), the contents of the dialyzing bags were analyzed for methyl green and nucleic acid phosphorus (the first was measured spectrophotometrically; the latter was obtained by two independent methods: (1) Allen's method (5) and (2) spectrophotometrically: since the absorption coefficient of a 1 mg./cc. DNA solution in 4.1 acetate buffer at 260 mμ is 20 and of methyl green it is 10 per cent of that at its maxi-

mum, the nucleic acid content of a DNA-methyl green solution in mg./cc.

$= \dfrac{E_{260} - .1E_{645}}{20}$). The methyl green in the sac was corrected for free methyl green by the concentration found in the bath. The results are given in Table XIV.

From Table XIV it is apparent that when the original ratio of dye/P_{DNA} molar was less than 0.099, the final ratio at equilibrium was within 10 per cent of the original value (indicating no significant loss of methyl green). With original mixtures of 0.099 dye/P_{DNA} or higher, the change is greater than 10 per cent. It is, therefore, our opinion that the mixture in which the dye/P_{DNA} was 0.099, represented approximate stoichiometric equivalence. If we consider the final dye/P_{DNA} values of all mixtures whose predialysis values fell by more than 10 per cent, we find values in the range 0.084 to 0.11 with an average of 0.095. This result is in agreement with those found on analysis of washed

TABLE XIV

Original mixture before dialysis Dye/P_{DNA}, molar	After dialysis Dye/P_{DNA}, molar	Original dye/P_{DNA}, molar	Final dye/P_{DNA}, molar
0.055	0.052	0.136	0.1
0.078	0.076	0.156	0.11
0.084	0.082	0.182	0.11
0.091	0.087	0.218	0.091
0.099	0.084		
0.109	0.09		
0.121	0.096		

stained precipitates, by measurement of the amount of dye removed from solution on precipitation of known amounts of DNA, and by extrapolation to zero of a curve of the amount of dye dialyzed from known mixtures of dye and nucleic acid.

As was noted in the preceding paper (1), solutions of DNA and methyl green were emerald green, whereas the dye alone gave blue-green solutions. When the DNA was in excess, so as to insure complete modification of the dye, the absorption maximum was found to have been shifted from 635 mμ to 645 mμ and the molecular extinction coefficient to have increased from 74,400 to 100,000. Whereas mixtures containing 0.1 dye molecule per P demonstrated the completely modified absorption spectrum in shape, the addition of DNA up to a ratio of 0.05 mol dye per P caused a slight increase in the absorption at 645 mμ (Text-fig. 3). Further increments of DNA had no measurable influence on either the shape or the height of the curve. At first sight, this observation would appear to contradict the stoichiometric relationship of 1 dye molecule per 10 P_{DNA}. However, the observations from the dialysis experiments that when the free methyl green concentration is kept at zero, continuous dissocia-

tion of the DNA-methyl green complex occurs, whereas when the free methyl green is not continuously removed, a stable equilibrium is reached, indicate that the reaction should be written:

$$10\ P_{DNA}\ +\ 1\ \text{methyl green}\ \rightleftarrows\ (P_{DNA})_{10}\ \text{methyl green}$$
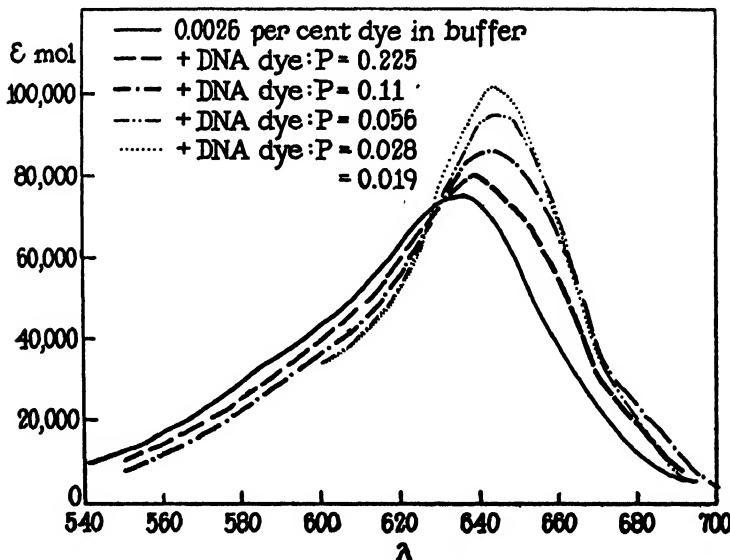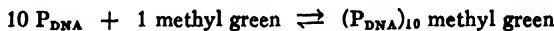
TEXT-FIG. 3. Effect of added DNA on absorption spectrum of methyl green.

TABLE XV

DNA Source	Dye/P 0.0128 per cent dye	Dye/P 0.05 per cent dye
Calf thymus chromosomes (saline).....................	0.075	0.081
Calf thymus chromosomes (sucrose)..................	0.079	0.089
Calf liver chromosomes............................	0.074	0.093
Shad sperm.......................................	0.069	0.086

Therefore, an excess of DNA would be expected to shift the reaction to the right, the concentration of free methyl green approaching zero as an asymptote. As a consequence, not all the methyl green is bound when the mixture contains the stoichiometric amounts of methyl green and DNA. When the DNA concentration exceeds twice the stoichiometric amount, the methyl green approximates 100 per cent bonding.

To determine whether or not preparations of DNA from several sources demonstrated the same stoichiometric relationship on staining with methyl

green, samples of DNA prepared from calf thymus chromosomes which had been isolated in saline, from thymus chromosomes isolated in 30 per cent sucrose, from shad sperm, and from beef liver chromosomes isolated in saline were compared (Table XV). The shad DNA appears to be more highly polymerized than the others since solutions of equal concentration are much more viscous.

In each case, solutions of the nucleic acid containing 0.3 mg. P_{DNA} were stained in 0.0128 per cent buffered methyl green and in 0.05 per cent buffered methyl green (final volume 4.8 cc.). After 2 hours, 0.2 cc. 1.5 per cent lanthanum acetate was added to each, the fibrous precipitates removed with a glass rod, and the suitably diluted supernates compared at 635 mμ (upon precipitation of the DNA, as has been mentioned (1), the solution reverts to the spectrum of methyl green alone) with controls containing no DNA. In each case, the dye/P, molar, found averaged 0.075 for the lower dye concentration and 0.089 for the higher concentration.

As has already been reported in the preceding paper (1), it is possible to determine the DNA content of isolated nuclei by measuring the amount of methyl green which they removed from a solution, since the DNA bound 1 molecule of dye per 10 P after removal of the histone with dilute HCl.

In a subsequent paper (4) a quantitative histochemical method for DNA based upon these stoichiometric observations will be reported.

CONCLUSIONS

Study of the stoichiometry of the DNA-methyl green reaction by dialysis, precipitation of stain-nucleic acid mixtures, and the staining of nuclei of known DNA content, indicate that the compound consists of one dye molecule per 10 P. The significance of this result was discussed in the preceding paper (1). Histone and lanthanum (and probably other multivalent cations (3)) compete with the dye for the nucleic acid molecule, indicating a common site of attachment, presumably the phosphoric acid groups.

With care in the avoidance of procedures which might depolymerize DNA, and the use of a buffer at about pH 4.1, a quantitative histochemical method for DNA by the use of methyl green is possible. Pyronin staining appears to be of qualitative significance only. Slight differences in degree of polymerization, as between the shad and mammalian DNA appear to have no effect on methyl green staining. It may be that a critical level of polymerization for DNA staining exists. This level must exceed 20 nucleotides to account for the 10 P to 1 dye molecule and the effect on the methyl green absorption spectrum; but it may be considerably greater. Beyond this critical level, whatever it may be, further polymerization probably has no influence on staining.

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MULTIPLICATION OF PNEUMONIA VIRUS OF MICE (PVM) IN THE RABBIT LUNG AND THE DEMONSTRATION OF A HEMAGGLUTINATING COMPONENT IN LUNG SUSPENSIONS FROM NORMAL ANIMALS

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During the course of experiments on the adaptation of pneumonia virus of mice (PVM) to the rabbit lung, it was found that a hemagglutinating component appeared in rabbit lung suspensions which had been heated and then stored for prolonged periods at 4 C. The close similarity of some of the *in vitro* reactions of this component, hereinafter referred to as RHC, to those of the virus seemed of sufficient interest to warrant detailed study. Hemagglutinating components have been demonstrated previously in the tissues of certain mammalian species. Stone (1) showed that lipid extracts from a variety of normal tissues agglutinate the same range of red blood cells as vaccinia and ectromelia virus hemagglutinins and that the activity of individual lipids was confined to the phospholipid group. Recently Salaman (2) reported that saline extracts of various mouse tissues agglutinate rabbit and mouse erythrocytes and showed that the hemagglutinating agent differs from viruses and lipids in important respects.

In the present communication the results of serial passage of PVM in the lungs of rabbits will be presented and it will be shown that the virus is capable of multiplication in this species. In addition, certain of the properties of a hemagglutinating component present in rabbit lung tissue will be described and compared with those of PVM.

Materials and Methods

RHC (rabbit hemagglutinating component). Rabbit lungs were ground for 2 minutes in a modified blendor in sufficient diluent to yield 10 per cent suspensions. The suspensions were heated at 70 C for 30 minutes and centrifuged in a field of 10,900 g for 30 minutes. The supernates developed hemagglutinating activity (*i.e.*, RHC) after storage at 4 C as described below.

PVM. Strain 15 (3) of the pneumonia virus of mice (PVM) was used exclusively. The virus was maintained by occasional lung passage in albino Swiss mice and stored as a 10 per cent suspension of infected mouse lungs at −70 C. "Heat released" virus suspensions (4) were prepared by treatment of suspensions of infected lungs in a manner identical to that described above.

Hemagglutination titrations. PVM and RHC: The technique of hemagglutina-

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tion titrations with mouse RBC, utilizing serial twofold dilutions, and the method of estimating end points were identical with those previously employed (5).

RBC suspensions. Blood was mixed with an acid-citrate dextrose solution (6) to yield a final concentration of 75 per cent. The mixture was stored at 4 C for periods not exceeding one week. As needed, the erythrocytes were washed 3 times in buffered saline and made up by volume to the desired concentration.

Solutions. Throughout this paper buffered saline refers to 0.15 M NaCl solution buffered at pH 7.2 with 0.01 M phosphate, and water refers to distilled water.

<div align="center">EXPERIMENTAL</div>

Passage of PVM in rabbit lungs. Serial passage of mouse lungs which harbor PVM as a latent agent yields virus suspensions which induce fatal pneumonia in mice, cotton rats and Syrian hamsters (7). Numerous laboratory stocks of each of these species are thought to carry PVM as a latent infection; the animals frequently exhibit neutralizing antibodies in their sera (7). Rabbit sera also often contain neutralizing antibodies against the virus, but adaptation of PVM to the lungs of rabbits has not been accomplished previously. Because one rabbit lung is equivalent in weight to approximately 100 mouse lungs, rabbit lungs infected with the virus should provide large volumes of PVM suspensions which would be less costly and more easily obtained than comparable quantities of mouse lung suspension.

Serial passage of PVM in 3-months-old rabbits was carried out. In each case rabbits were employed only after it was shown that their serum contained no demonstrable hemagglutination-inhibiting antibody against the virus. Five ml of a 10^{-2} dilution of infected mouse lung suspension (*i.e.*, $10^{3.6}$ M.S.50 doses (8) for mice) were inoculated intratracheally into each of 2 lightly anesthetized rabbits. After 6 days the animals were killed with chloroform and their lungs were removed, weighed and ground for 3 minutes in a modified blendor at 4 C in sufficient broth to yield a 50 per cent suspension. One aliquot was diluted with water to a 10 per cent suspension, ground for one additional minute in the blendor, heated at 70 C for 30 minutes, and the hemagglutination titer of released virus measured with mouse RBC. Another aliquot was diluted with broth to a 10 per cent suspension, ground for an additional 15 seconds and lightly centrifuged to yield a virus suspension for further passage. Additional rabbits were inoculated intratracheally in series with the rabbit lung suspension which showed the highest hemagglutination titer. In some instances suspensions of two lungs were pooled for subinoculation. Usually a 5 ml inoculum of a 10^{-1} suspension was employed. In the third passage a 10^{-2} dilution was used and in passages 6 to 10 between 6 and 8 ml of a 10^{-1} suspension were inoculated. At each passage the inoculum was also given intranasally to a

group of 6 mice, and the lung lesion score determined in the usual manner (8).
In most instances the hemagglutination titer of heat-released virus was meas-
ured in the lungs of another group of mice 6 days after a similar inoculation.
Three control passages were carried out in normal rabbits. In the first passage
5 ml of a 10^{-2} dilution of normal mouse lung suspension were inoculated. In the
second and third passages a similar quantity of a 10^{-1} dilution of rabbit lung
suspension was given.

TABLE I

Results of Serial Passage of PVM in Rabbit Lungs

Passage No.	Rabbit lung suspension*				Results of subinoculation of passage material in mice	
	Hemagglutination titer vs. mouse RBC				Lung lesion Score‡ 12th day	Hemagglutination titer—6th day vs. mouse RBC
	Rabbit No.†					
	1	2	3	4		
1	32	16	0		21/30	—
2	32	16			30/30	64
3	64	8			25/30	64
4	32	0	0	0	25/30	—
5	0	0			1/30	32
6	0	0			0/30	64
7	32	8			30/30	256
8	0	0			0/30	16
9	64	16			14/30	32
10	128	32			30/30	—
11	128	0	0	0	11/30	512
12	0	0			—	0

* Suspension heated at 70 C for 30 minutes, and centrifuged at 10,000 g.

† Values underlined indicate unheated suspensions employed for passage.

‡ Numerator equals observed lung lesion score; denominator equals maximum possible
lung lesion score.

The results are recorded in Table I. It will be seen that, although PVM was
carried successfully through 11 serial passages in rabbit lungs, the results
obtained in individual animals were irregular, and in only a few instances were
hemagglutination titers of 1:64 or more obtained. In both the 10th and 11th
passages individual rabbit lung suspensions gave titers of 1:128. The former
was also tested for infectivity in mice and showed a virus titer (M.S.50) of
$10^{-3.2}$. Specific anti-PVM serum inhibited hemagglutination and neutralized
the infectivity of the agent in mice. Control passages in normal rabbits failed
to reveal the presence of the virus. It appears, therefore, that the virus is
capable of multiplication in rabbit lungs. However, there was no clear indica-
tion that the pathogenicity of the agent for this species increased on prolonged

serial passage; the virus titer did not increase markedly over that obtained in the first passage, and significant lung lesions did not develop.

Hemagglutinating component in rabbit lungs. It will be noted that after 3 successful serial passages of PVM in rabbit lungs the amount of virus obtained in 3 subsequent passages diminished markedly. To exclude the possibility that an error had been made in the measurement of virus titer of the suspensions employed in the early passages, the hemagglutination titers of heated suspensions were determined again after storage at 4 C for 21 to 28 days. Surprisingly, the titers were found to be 10 to 20-fold higher than those originally observed with the same heated suspensions. That the final high hemagglutination titers were not attributable to the virus became evident when it was found that PVM immune sera failed to inhibit the hemagglutination reaction. Apparently, therefore, the hemagglutination titers obtained with heated rabbit lung suspensions, which had been stored for 3 to 4 weeks at 4 C, were due to the appearance of a component other than PVM in the suspensions. For convenience, this component has been designated RHC. The conditions which influence the appearance of this component are defined below.

Appearance of RHC at 4 C. A number of rabbit lung suspensions were prepared in saline or water and heated at 70 C for 30 minutes. The suspensions were tested for hemagglutinating capacity against mouse RBC immediately after preparation and at intervals after storage at 4 C. In eleven instances the hemagglutination titer obtained immediately after the suspensions were prepared was zero. The results obtained with these suspensions after storage are shown in Fig. 1. The logarithms of the highest, lowest and geometric mean hemagglutination titer are plotted against time of storage at 4 C. It will be observed that, although RHC was not demonstrable in freshly prepared heated rabbit lung suspensions, it appeared upon storage at 4 C and reached a maximal titer at approximately 18 days. `

Stability of RHC at 4 C. Nine RHC preparations were kept at 4 C and their hemagglutination titers against mouse RBC were determined at weekly intervals for 2 months. In Fig. 1 the results of these titrations also are presented graphically. It is evident that no significant decrease occurred in the titers of the RHC preparations on prolonged storage at 4 C.

Frequency of appearance of RHC. Although RHC had been observed to develop in a number of heated rabbit lung suspensions, it seemed desirable to increase the number of observations in order to determine whether or not the appearance of RHC was characteristic of such suspensions. Heated lung suspensions were prepared from 37 rabbits which had been inoculated either with PVM or normal rabbit lung suspensions as well as from 7 normal rabbits, and these were examined before and after 3 or more weeks' storage at 4 C. In Table II the initial and the highest hemagglutination titers obtained with each preparation are recorded. Although RHC was not demonstrable initially in 11

of 12 heated suspensions of normal or control rabbit lungs, it is evident that it appeared in all of the suspensions after prolonged storage at 4 C. All but 3 of the suspensions developed hemagglutination titers of 1:128 or greater. It is evident also that in heated suspensions of the lungs of rabbits inoculated with
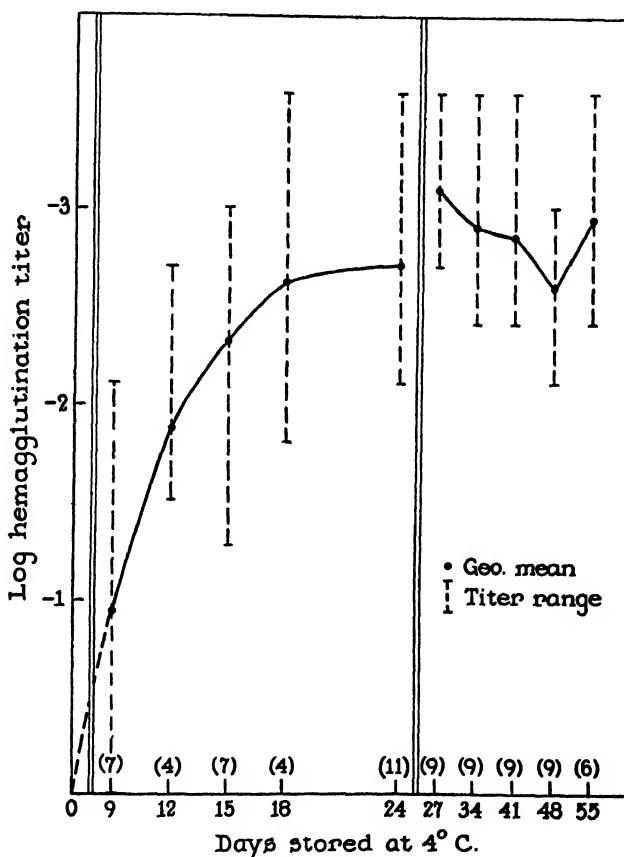
Fig. 1. Increase in hemagglutination titer of heated rabbit lung suspensions during storage at 4 C. The number of suspensions tested at each interval is given by the figure in parenthesis.

PVM, this hemagglutinating component appeared on storage at 4 C. All but 6 of 32 such suspensions developed titers of 1:128 or more.

Effect of temperature before storage on RHC. The development of RHC was not prevented by prolonged heating prior to storage. Aliquots of 2 normal rabbit lung suspensions were heated at 70 C for 12 hours and then boiled for 15 minutes. After storage at 4 C for some weeks the hemagglutination titer of these suspensions was equal to that of other aliquots which had been heated at

70 C for only 30 minutes and stored for a similar period. In contrast, it was found that with 11 rabbit lung suspensions which were frozen at −30 C for some weeks prior to heating, RHC did not develop even though the preparations were held for 4 months at 4 C.

Hemagglutination with other mammalian lung suspensions. In order to determine whether the lungs of other mammalian species also would show the appearance of hemagglutinating component under similar conditions, 10 per cent suspensions of normal mouse, guinea pig and hamster lungs were prepared in saline, heated and centrifuged as before, and then stored at 4 C for 3 weeks. The supernates were tested by the hemagglutination technique with mouse RBC immediately after preparation and after 3 weeks' storage at 4 C. In all instances hemagglutination was not demonstrable with the original suspensions. The hamster lung suspension developed a hemagglutinating titer of 1:512

TABLE II

Maximum Hemagglutination Titers of Heated Rabbit Lung Suspensions after Storage at 4 C

Rabbit lung suspensions	No.	Initial hemagglut. titer vs. mouse RBC						Highest hemagglut. titer after 3 or more weeks' storage at 4 C. vs. mouse RBC											
		No. of rabbits which showed indicated titer																	
		0	4	8	16	32	64	0	4	8	16	32	64	128	256	512	1024	2048	4096
Inoculated with PVM	32	17	2	2	1	4	1	0	0	0	1	2	3	2	2	8	8	4	2
Control	5	4	0	1	0	0	0					1		1			1	1	1
Normal	7	7	0	0	0	0	0			1		1		1	1	1		2	
Total	44	28	2	3	1	4	1	0	0	1	1	4	3	4	3	9	9	7	3

after storage. One guinea pig lung suspension showed a titer of 1:512 after storage and two others gave titers below 1:16. None of the mouse lung suspensions developed a hemagglutinating component on storage at 4 C.

Agglutination of various erythrocytes by RHC. Erythrocytes from various animal species were tested with both PVM and RHC in the following manner: Serial twofold dilutions of the virus or of RHC were made in saline. To each dilution was added an equal volume of 0.8 per cent RBC from the desired species. After 90 minutes at room temperature the presence or absence of hemagglutination was determined and the titer recorded. The results are recorded in Table III. It will be seen that both PVM and RHC in high dilution agglutinated erythrocytes obtained from mice or hamsters but did not agglutinate those of any other species employed. It was found that with RHC, as with PVM (9), hemagglutination did not occur when the electrolyte concentration was 0.01 M or less in solutions made isotonic by the addition of glucose.

Combination of RHC with lung tissue. A striking property of PVM is its ability to combine with lung tissue particles obtained from certain mammalian

species (5, 10). To determine if RHC would also combine with lung tissue particles, the following experiment was performed: 10 per cent suspensions of normal rabbit and perfused normal mouse lungs were prepared in the usual manner and to each an equal volume of PVM or RHC was added. The mixtures were held for 30 minutes and the lung particles were then sedimented by centrifugation. The hemagglutination titer of each supernate was determined.

TABLE III

Agglutination of RBC from Various Species by RHC and PVM

Erythrocytes	Hemagglutination titer	
	RHC	PVM
Mouse..................................	512	512
Hamster.................................	256	1024
Chicken..	0	0
Human (Group O).................................	0	0
Guinea Pig.......................................	0	0
Sheep..	0	0
Cotton Rat.......................................	0	0

TABLE IV

Inhibition of Hemagglutination with RHC and PVM by Tissue Suspensions

Mixture		Treatment		Hemagglutination titer of supernate vs. Mouse RBC
Agent	Tissue suspension	Held for 30 min. at	Centrifuged 30 min. at	
	10 per cent	°C	*× g.*	
RHC	Mouse lung	22	10,600	0
	Rabbit lung	22	10,600	8
	Saline	—	—	256
PVM	Mouse lung	36	15,000	0
	Rabbit lung	36	15,000	0
	Saline	—	—	256

The results shown in Table IV indicate clearly that RHC, like PVM, combines with lung tissue particles obtained either from the rabbit or the mouse.

This finding suggested that RHC might combine with the same lung tissue component which combines with PVM. If this were the case, it seemed possible that the instillation of RHC into the mouse lung might prevent the multiplication of PVM inoculated subsequently, by blocking adsorption of the virus by susceptible cells. It was found that no inhibition of multiplication could be demonstrated. Even though mice were repeatedly given considerable amounts of RHC intranasally, they remained fully susceptible to infection with PVM.

Inhibition of RHC with serum. Hemagglutination by RHC is inhibited by nor-

mal serum (heated at 56 C for 30 minutes) obtained from guinea pigs, mice, rabbits or hamsters. Such sera inhibit hemagglutination by 8 units of RHC in dilutions as high as 1:200. The inhibiting component in serum appears to be heat labile and is inactivated by heating at 65 C for 30 minutes. Attempts to produce antibodies against RHC in rabbits, guinea pigs and mice were unsuccessful. Despite the repeated intraperitoneal injection of high titer material, the serum of immunized animals consistently failed to show the presence of specific antibodies against the component. Inhibition of hemagglutination obtained with such sera was not demonstrably different from that shown by normal sera.

Other properties of RHC. Numerous cultures, both aerobic and anaerobic, of RHC preparations of high hemagglutination titer usually failed to demonstrate the presence of bacteria. Occasionally an obvious contaminant was obtained. RHC was dialyzed in cellophane against large volumes of buffered saline and was centrifuged in a field of 10,900 g for 30 minutes without causing any detectable change in the hemagglutination titer of the preparation. The active component was filterable through porcelain candles (Coors P_2, P_3 and Selas 0.03) without significant loss in titer. After RHC had developed in a lung suspension it was relatively unstable on heating. Three to 6 hours at 37 C or 30 minutes at 70 C resulted in a decrease in hemagglutination titer equal to the inactivation of 75 and 90 per cent, respectively. The component was precipitated in half saturated $(NH_4)_2SO_4$ solution at pH 7.0 and was recovered in undiminished concentration when the precipitate was resuspended in buffered saline. The component was inactivated by 50 per cent methyl alcohol at both room temperature and 4 C and all attempts to extract it with alcohol-ether mixtures, acetone or chloroform at room temperature were unsuccessful.

Effect of enzymes on RHC. In an attempt to learn something of the nature of the active component, preparations of RHC of high hemagglutination titer were treated with various enzymes at 37 C for 1 to 2 hours. For comparative purposes heat-released suspensions of PVM were treated with the same enzymes under identical conditions. The hemagglutination titer of each mixture after incubation was determined with mouse RBC and compared with that of an appropriate saline control. The results are recorded in Table V. None of the proteolytic enzymes employed inactivated RHC. However, both cobra venom and alpha toxin of *C. welchii* caused marked reduction in the hemagglutination titer of the active component.[1] It will be noted that in the presence of sodium citrate alpha toxin had no effect upon RHC. It is of interest that both cobra

[1] Crystalline trypsin and chymotrypsin were kindly provided by Dr. Maclyn McCarty, Rockefeller Institute, N. Y. Alpha toxin *Cl. welchii* was made available through the courtesy of Dr. Mark H. Adams, New York University College of Medicine, N. Y. Cobra venom was obtained from Ross Allen's Reptile Institute, Silver Springs, Fla.

venom and alpha toxin contain enzymes which act on lipids, especially lecithin (11, 12), and that in the absence of ionized calcium this enzyme of alpha toxin is inactive. In the concentrations which were employed in experiments with RHC neither cobra venom nor alpha toxin had any demonstrable direct effect upon mouse RBC themselves. Erythrocytes treated with either cobra venom or alpha toxin remained agglutinable by high dilutions of RHC.

TABLE V

Effect of Various Enzyme Preparations on RHC and PVM

Agent	Enzyme preparation		Mixture held at 37 C.	Hemagglutination titer vs. mouse RBC	
	Kind	Final concentration per ml		Agent + saline	Agent + enzyme prep.
			min.		
RHC	Trypsin, crystalline	0.5 mg	120	512	512
	Chymotrypsin, crystalline	0.5 mg	120	512	512
	Papain, activated*	1.0 mg	120	512	1024
	Pancreatin	1.0 mg	120	512	512
	α toxin *Cl. welchii*†	3 MLD	120	1024	16
	α toxin *Cl. welchii* + Na citrate 1.75%	3 MLD	120	1024	1024
	Cobra venom	0.001 mg	60	1024	32
PVM	Trypsin, crystalline	0.5 mg	120	256	32
	Chymotrypsin, crystalline	0.5 mg	120	256	64
	Papain, activated*	1.0 mg	120	256	256
	Pancreatin	1.0 mg	120	256	32
	α toxin *Cl. welchii*†	3 MLD	120	256	256
	Cobra venom	0.2 mg	120	256	64

* Contains 0.003 M neutral thioglycolic acid.

† Buffered with borate, pH 7.2, containing $CaCl_2$ as recommended by Adams (13). MLD determined in mice.

In confirmation of earlier findings (5) the hemagglutination titer of PVM was reduced by the action of crystalline trypsin or chymotrypsin and also by commercial pancreatin. Surprisingly, PVM was unaffected by activated papain. Cobra venom in a concentration of 0.2 mg per ml caused a definite reduction in the titer of the virus but alpha toxin had no effect. It seems probable that the action of cobra venom on PVM can be ascribed to a proteolytic enzyme.

DISCUSSION

A peculiar characteristic of the hemagglutinating component of rabbit lung (RHC) is evidenced by its apparent absence in freshly prepared suspensions and its gradual appearance in heated cell-free supernates during prolonged storage at 4 C. The mechanism by which the component becomes demonstrable has not

been revealed in this study. The following possibilities arise: 1) The component may arise *de novo*, *i.e.*, appear as a result of synthetic processes which proceed during storage in the cold. 2) An inactive precursor of the component may be present in fresh suspensions and the active component appear only after the hypothetical precursor has been altered during storage. 3) The gradual degradation of constituents of the lung tissue may result in the development of a component which has hemagglutinating activity. Whatever the actual mechanism may be, it is apparent that it is not dependent upon substances which are readily denatured by heat. It should be emphasized that RHC developed in suspensions which had been treated at 70 C for 30 minutes.

Somewhat similar findings with chick embryo membranes have been reported by Stone and Burnet (14). They found that in saline emulsions of material from vaccinia virus lesions on the chorioallantoic membrane, a hemagglutinating component developed upon storage at 4 C for 10 days or more. This component was distinct from the virus "hemagglutinin", caused agglutination of certain fowl and mouse erythrocytes, and was inhibited by normal serum in high dilution. Lipid solvent extracts of chorioallantoic membranes and chick embryo liver, as well as mixtures of certain purified lipids, exhibited similar hemagglutinating properties (1, 15). These workers suggested that the development of a hemagglutination component in saline emulsions of chorioallantoic membranes upon storage in the cold was due to the liberation of lipid material by autolytic processes (14).

That a lipid constituent is associated with the activity of RHC is indicated by the finding that the hemagglutinating activity of the component is rapidly destroyed by preparations containing lecithinase A or C. This result is analogous to the destruction of vaccinia and ectromelia virus "hemagglutinins" by the same enzymes which was reported by Stone (16). However, the failure to extract a hemagglutinating component from freshly prepared heated rabbit lung suspensions with lipid solvents, the inactivation of RHC by cold alcohol, and the heat lability of the developed component suggest that RHC is not composed solely of lipid. It seems more probable that RHC is a complex substance which contains phospholipid, and that the presence of lipid is essential to its hemagglutinating activity. The other constituents which are as yet unidentified appear to be of large molecular size, since the component was nondialysable, and may be protein or carbohydrate in nature.

The hemagglutinating component of rabbit lung differs from the hemagglutinins extractable from various tissues with organic solvents, as reported by Burnet and Stone (1, 15) in that it does not agglutinate chicken RBC, is heat labile after development, is not extracted by organic solvents, and appears only after prolonged storage at 4 C of heated suspensions. RHC also differs from the hemagglutinin in freshly prepared saline extracts of mouse tissues as reported by Salaman (2) in that the former agglutinates only mouse and ham-

ster RBC, is rapidly destroyed by lecithinases but not by proteolytic enzymes, is stable at 4 C, and is inhibited by normal serum. Moreover, RHC differs from the hemagglutinin in the globulin fraction of egg white (17) in that it agglutinates a different species range of RBC, develops on storage at 4 C, and is more thermolabile after development.

SUMMARY

Multiplication of PVM in the rabbit lung after intratracheal inoculation has been demonstrated. The virus was maintained through eleven serial passages in rabbit lungs. The development of a hemagglutinating component (RHC) in heated suspensions of rabbit lungs upon prolonged storage at 4 C is described and various properties of the component are enumerated. There is a close similarity between the hemagglutination reactions of RHC and PVM. The differences between RHC and hemagglutinating components previously found in other animal tissues are discussed. The available evidence suggests that RHC is a complex substance of large size which contains phospholipid and indicates that the lipid is essential for hemagglutinating activity.

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FURTHER STUDIES ON THE ASSOCIATIVE REACTIONS OF PNEUMONIA VIRUS OF MICE (PVM) AND INFLUENZA VIRUSES

Combination with Various Animal Tissues and Adsorbents

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In earlier studies (1–6) it was demonstrated that pneumonia virus of mice (PVM) combines with erythrocytes of the mouse or hamster as well as with tissue components present in the lungs of certain mammalian species. With the methods employed a similar component capable of combining with the virus could not be demonstrated in any tissue of avian species studied nor in organs, other than the lungs, of mammals susceptible to infection with (PVM) (5). Moreover, although multiplication of the virus occurs in the lung tissues of susceptible mammalian species (7–9), attempts to infect avian embryos or extraembryonic tissues with PVM as well as to demonstrate multiplication of the virus in non-pulmonary tissues of infected mammals have been uniformly unsuccessful (2, 4, 5, 7). These findings indicated that, with the notable exception of mouse or hamster erythrocytes, there was an almost complete correlation between the presence of components which combine with the virus in a given tissue and the capacity to support multiplication of the agent. On this basis the hypothesis was considered (2, 4, 5) that the lung tissue component may be an important factor in the pathogenesis of pulmonary infection with PVM and that susceptibility to infection with the virus may be in part dependent upon the presence of a tissue component with which the virus can combine.

During the course of an investigation on the nature of the virus-combining component in lung tissue, methods were developed which were more delicate than those employed previously to measure the concentration of the component. When other tissues were reexamined with the aid of these techniques, it was found that a wide variety of organs contain tissue components which combine with PVM. Moreover, it was found that the virus can combine with certain adsorbents and then can be eluted from them. Adsorption and elution of influenza viruses also were demonstrated with the same adsorbents.

Materials and Methods

PVM.—Strain 15 of pneumonia virus of mice (PVM) (7) was used exclusively. It was maintained by occasional lung passage in albino Swiss mice and was stored as a 10 per cent

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suspension of infected mouse lungs at −70°C. Heat-released virus suspensions in distilled water were prepared as previously described (6) and were stored at 4°C. Prior to use, sufficient NaCl to yield a concentration of 0.15 M was added.

Influenza Viruses.—The PR8 strain of influenza A virus and the Lee strain of influenza B virus were used. They were maintained by passage in the allantoic sac of chick embryos.

Hemagglutination Titrations.—The technique of hemagglutination titrations with mouse or chicken RBC, utilizing serial twofold dilutions, and the method of estimating end points were identical to those previously employed (6).

RBC Suspensions.—Blood from mice or chickens was mixed with an acid-citrate dextrose solution (10) to yield a final concentration of 75 per cent. The mixtures were stored at 4°C. for periods not exceeding 1 week. As needed, the erythrocytes were washed 3 times in buffered saline and made up by volume to the desired concentration.

Normal Tissue Suspensions.—Normal mice, rabbits, or chickens were selected which possessed no demonstrable antibodies against PVM in their serum. After anesthetization the vascular system of mice was perfused with saline to free the tissues of as much blood as possible. Perfusion was not carried out with rabbits or chickens. Various organs were removed from each animal at autopsy and separate organs were ground in buffered saline to 10 per cent suspensions by weight in a modified Waring blendor. Grinding was continued for 2 minutes and the blendor was constantly cooled in an ice bath. Tissue suspensions were stored at −28°C. until used.

Adsorbents.—Various commercial adsorbents, enumerated below, were employed. Each adsorbent, in a concentration of 10 per cent by weight, was suspended in buffered saline and washed 3 times by centrifugation.

Solutions.—Throughout this paper buffered saline refers to 0.15 M NaCl solution buffered at pH 7.2 with 0.01 M phosphate, and water refers to distilled water. All pH values were determined with a Beckman pH meter.

<div align="center">EXPERIMENTAL</div>

Virus-Combining Capacity of Lung Tissue.—In early studies (1, 2) the capacity of certain tissues to combine with PVM was demonstrated in mixtures containing relatively large amounts of both tissue and virus. Determination of the amounts of virus in the supernate and in the sediment from such a mixture gave some indication of the combining capacity of the tissue but did not permit satisfactory quantitation. Subsequently (5) a more precise procedure was devised; serial dilutions of a tissue suspension were mixed with constant small amounts of virus and the presence of free virus in the supernates was determined. The virus-combining titer of a tissue suspension could be determined in this manner. It was found (5) that, among various tissues studied with this procedure, only mammalian lung tissue suspensions showed a demonstrable capacity to combine with PVM. Employing the same procedure (5) evidence was obtained that the virus and mouse lung tissue combined in stoichiometric proportions. It should be pointed out that these studies of the capacity of various tissues to combine with PVM were carried out under definite experimental conditions; all mixtures contained 0.15 M NaCl; all supernates were obtained after centrifugation at 10,900 *g.* for 10 minutes (equivalent to 109,000 *g.* minutes).

Recently it was shown (6) that the virus-combining titer of normal mouse lung suspensions was directly related to the NaCl concentration of the mixtures. The results of various experiments carried out during the present study raised the possibility that the amount of centrifugation also might influence the result. This hypothesis was tested in the following manner:

To each of a series of twofold dilutions of a suspension of perfused normal mouse lungs in 0.15 M NaCl, 16 hemagglutinating units of PVM in 0.15 M NaCl was added. Following incubation at 37°C. for 30 minutes the mixtures were centrifuged and the supernates were tested for free virus by the hemagglutination technique with mouse RBC. The amount of centrifugation was varied over a wide range but other variables were kept constant. Mixtures of virus and 0.15 M NaCl solution were centrifuged simultaneously to control sedimentation of uncombined virus. With the amounts of centrifugation used there was no significant sedimentation of free PVM.

TABLE I

Effect of Centrifugation on PVM-Combining Titer of Normal Mouse Lung Suspension

Mixtures		Centrifugation			Agglutination with supernates vs. mouse RBC										PVM-combining titer of suspension
Normal mouse lung suspension in 0.15 M NaCl	Virus added to each dilution	Gravitational force	Time	Amount of centrifugation	Dilution of mouse lung suspension										
					16	32	64	128	256	512	1024	2048	4096	8192	
	units	*g.*	*min.*	*g. min.*											
Serial dilutions	16	10,900	10	109,000	0	1	2	3	3						16
" "	16	10,900	15	163,000	0	0	0	2	3						64
" "	16	15,500	10	155,000	0	0	0	0	2	3	3	3			128
" "	16	15,500	30	465,000	0	0	0	0	±	3	3	3	3	3	256
" "	16	15,500	60	930,000	0	0	0	0	0	0	0	±	3	3	2048

The results of a typical experiment are shown in Table I. It is evident that as the amount of centrifugation (the product of the gravitational force and time which is expressed in g. minutes) was increased, the apparent virus-combining titer of the suspension also increased. Because it is obvious that the actual capacity of the tissue to combine with virus could not be influenced by the amount of centrifugation, it is apparent that other factors were responsible for the results.

It will be recalled that in quantitative sedimentation studies (3) with mixtures of normal mouse lung suspensions and PVM, it was shown that when 92,100 g. minutes of centrifugation was employed approximately 30 per cent of the combined virus remained in the supernate. The addition of mouse RBC to such a supernate provides another component with which the virus can combine. If the attractive forces between lung tissue and virus are of the same order as those between RBC and virus, as studies (6) with varying electrolyte

concentrations suggest, it would be expected that when all three components are present RBC-virus combination, *i.e.* hemagglutination, should occur when RBC concentration exceeds lung tissue concentration. In other words, if RBC and lung tissue compete for virus, the result will be determined by the ratio of the concentrations of the combining components.

On this basis, the effect of the amount of centrifugation on the virus-combining titer is readily understood. Increasing amounts of centrifugation result in the sedimentation of increasing quantities of combined virus. As progressively less combined virus remains in the supernate, less virus is available for transfer to added RBC and hemagglutination fails to be demonstrable. The net effect is an apparent increase in virus-combining titer which is dependent upon a decrease in combined virus in the supernate. It is apparent from these results and those obtained previously (6) that both the demonstration of PVM-tissue combination and the measurement of virus-combining capacity are markedly influenced by the experimental conditions employed.

Virus-Combining Capacity of Various Tissues.—Because of the findings discussed in the preceding section, it appeared important to devise a more satisfactory technique for the study of PVM-tissue combination and to employ it in an investigation on a variety of tissues from various animal species. The procedure which was developed for this study takes advantage of the recently established facts (6) that PVM-tissue component complexes can be washed repeatedly in the presence of 0.15 M NaCl without undergoing dissociation and that dissociation occurs when the electrolyte concentration is reduced to 0.01 M or less.

Ten per cent suspensions in 0.15 M NaCl were prepared from a variety of tissues obtained from normal mice, rabbits, and chickens as described above. One cc. of each suspension was centrifuged at 4,850 *g*. for 5 minutes (24,250 *g*. minutes). The supernate was poured off and the sedimented tissue particles were resuspended in 1 cc. of a 10 per cent suspension of heat-released PVM. The mixture was held at 37°C. for 30 minutes and then centrifuged at 4,850 *g*. for 10 minutes (48,500 *g*. minutes). The supernate was removed, and the concentration of free virus was measured by the hemagglutination technique. The sediment was resuspended and washed twice in 5 cc. of buffered saline with centrifugation of 48,500 *g*. minutes at each step. The supernate from the second washing was tested by the hemagglutination technique to determine the presence of any free virus. Almost without exception there was no demonstrable virus in this supernate; in occasional instances titers of 1:4 were obtained. The washed sediment was resuspended in 1 cc. of distilled water, held for 30 minutes at 23°C., and centrifuged 327,000 *g*. minutes. Two-tenths cc. of the supernate was removed and the concentration of free virus was measured by the hemagglutination technique. The sediment was then resuspended in the supernate, heated at 70°C. for 30 minutes, and centrifuged 72,800 *g*. minutes. The hemagglutination titer of this supernate was also determined. In each experiment a suspension of heat-released PVM and saline was carried through all steps in the procedure as a control. In addition, a suspension of each normal tissue in saline was carried through all steps of the procedure.

The results of typical experiments are recorded in Table II. It will be seen that with perfused tissues of normal mice, sedimentable particles derived from

either lung or heart muscle combined with large amounts of PVM. Sedimentable particles obtained from all other mouse tissues tested were also capable of combining with the virus although in smaller amount. Particles obtained from ground mouse RBC, in contrast to intact RBC (2), combined with relatively small amounts of virus. With tissues of normal rabbits, particles

TABLE II

Combination of PVM with Sedimentable Particles Derived from Various Animal Tissues

Mixture of PVM and sedimented particles from		Hemagglutination titer of supernate	Amount of virus* adsorbed from supernate	Hemagglutination titer of supernate from washed particles resuspended in H₂O	Hemagglutination titer of supernate from washed particles heated 70°C. for 30 min.	Amount of virus* released from washed particles
Species	Tissue					
			units			*units*
Control mouse	Saline	512	0	0	0	0
	Lung	8	1260	128	128	320
	Heart	64	1120	128	256	640
	Liver	256	640	32	64	160
	Spleen	512	0	32	64	160
	RBC	512	0	8	16	40
	Kidney	512	0	16	32	80
	Muscle	512	0	4	16	40
Control rabbit	Saline	256	0	0	0	0
	Lung	32	560	256	256	640
	Heart	64	480	128	128	320
	Liver	64	480	256	256	640
	Spleen	256	0	64	128	320
	RBC	128	320	64	64	160
	Kidney	256	0	16	32	80
	Muscle	256	0	0	0	0
Control chicken	Saline	256	0	0	0	0
	Lung	256	0	0	0	0
	Heart	256	0	16	32	80
	RBC	256	0	0	0	0
	Muscle	256	0	0	0	0

* Computed on the basis that 1.0 cc. of virus was added to each preparation of tissue particles.

derived from lung, heart muscle, and liver combined with considerable quantities of the agent. Rabbit skeletal muscle was the only tissue tested which failed to yield particles with some virus-combining capacity. With the exception of particles from chicken heart muscle which showed virus combination in minor degree, tissue particles obtained from normal chickens possessed no demonstrable combining capacity.

It should be emphasized that in every instance tissues were obtained only

from animals which possessed no demonstrable antibodies against PVM in their serum. Moreover, in the case of mice the vascular system was perfused with large amounts of saline to remove as much blood as possible from the tissues. That the small quantity of RBC which remained in the tissues could account for the combination which occurred with various tissues is highly improbable in view of the results obtained with ground RBC themselves. Although stromata of mouse RBC combine with PVM (2), the technique employed in this study does not effectively sediment such stromata and during successive washings of the sediment they are discarded with the supernates.

That the hemagglutinating component which was released from the washed and resuspended tissue particles was in fact the virus is evident for a number of reasons. The component was not demonstrable in the supernates until the sediments were resuspended in distilled water, a procedure which results in dissociation of PVM from combination (6). Heating the released component at 70°C. for 30 minutes followed by 72,800 g. minutes of centrifugation caused no reduction in the hemagglutination titer which is consistent with earlier results obtained with PVM (2, 3, 6). In no instance was a hemagglutinating component released from control tissue particles which were mixed with saline and carried through the entire procedure.

The results of these experiments are, in a number of instances, different from those obtained in previous studies (2, 5) which indicated that among mammalian organs only the lung contained a component capable of combining with PVM. The development of a more delicate technique for determining the occurrence of combination appears to have been responsible for the present demonstration that tissue particles derived from numerous organs of the mouse and rabbit are capable of combining with the virus in some degree.

Combination of PVM with Adsorbents.—Because PVM can combine and be sedimented with particles derived from a wide variety of animal tissues, it appeared unlikely that some special tissue component is necessary for such combination to occur. More probable seemed the possibility that the virus was adsorbed by tissue particles in a physicochemical sense, and was eluted from them when the environment was altered, as by reducing the electrolyte concentration (6), increasing the pH (4), or upon heating (2). If this hypothesis were correct, it should be possible to demonstrate adsorption of the agent with one or another of the adsorbents commonly used in physicochemical studies. A number of experiments were carried out as follows:—

Adsorbents which are available from commercial sources, such as potato starch, various grades of celite and amberlite IRA-400, an ion exchange resin, were employed. The adsorbent was washed 3 times in buffered saline in the centrifuge and the final supernate was discarded. Two cc. of a suspension of heat-released PVM in 0.15 M NaCl at pH 6.8 was added to the packed adsorbent. The mixture was agitated by tilting to and fro for 10 minutes. The adsorbent then was sedimented in the centrifuge and the concentration of virus remaining in the supernate was measured by the hemagglutination technique.

As is shown in Table III, definite adsorption of PVM by celite 503, hyflo-super cel, potato starch, or amberlite IRA-400 was not demonstrable. However, adsorption of 90 per cent or more of the virus by super-cel, celite 505, filter cel, or analytical filter-aid was readily demonstrated. It is noteworthy that adsorption of PVM by various celites was obtained only with those grades which give slow liquid flow rates, a finding which is correlated with reduced particle size and increased total surface area of the adsorbent.

It was to be expected that the attractive forces which bind PVM to celite adsorbents could be influenced by changing the ionic environment and the pH. It was found that partial elution of the virus from celite could be achieved by raising the pH of the mixture to 9 or more with buffers.

TABLE III

Adsorption of PVM on Various Adsorbents

Mixture PVM and adsorbent		Hemagglutina- tion titer of supernate
	gm.	
Saline..	—	512
Celite 503...	0.5	256
Hyflo super-cel...	0.5	256
Super-cel..	0.5	32
Celite 505...	0.5	64
Filter-cel..	0.5	0
Analytical filter-aid...	0.5	8
Saline..	—	256
Potato starch..	1.0	128
Amberlite IRA-400...	1.0	128

One-half gm. aliquots of celite analytical filter-aid were washed 3 times in buffered saline in the centrifuge, and the final supernates discarded. Two cc. of a suspension of heat-released PVM in 0.15 M NaCl at pH 6.8 was added to the packed adsorbent. The mixture was agitated by tilting to and fro for 10 minutes. The adsorbent was sedimented in the centrifuge and the concentration of virus in the supernate measured by the hemagglutination technique. The adsorbent was then washed twice in 5 cc. of buffered saline and resuspended in 0.1 M buffer solution of the desired pH. In no instance was virus demonstrable in the supernate from the last washing. The mixture was equilibrated by agitation for 10 minutes and then centrifuged. The concentration of virus in the buffer supernate was measured as before. Each buffer solution used was shown to be incapable of agglutinating mouse cells.

The results are recorded in Table IV. It will be seen that, although approximately 90 per cent of the virus was adsorbed by the grade of celite employed, no elution was demonstrable at pH 8 or below. At pH 9 and above, elution of approximately 10 per cent of the adsorbed virus was obtained. That elution was due to the alkaline pH and not to an ionic effect of the NH₃-NH₄Cl buffer

is indicated by the fact that glycine-NaOH buffers at pH 9 or 10 also caused elution of the virus in similar amount. That the hemagglutinating component released with alkaline buffers was actually the virus was established by specific serological identification of the agent with anti-PVM immune mouse serum. It is of interest that elution of PVM from celite at alkaline pH is analogous to dissociation of combined virus from lung tissue particles at pH levels of 10 or higher (4).

Combination of Influenza Viruses with Adsorbents.—Adsorption of influenza virus (PR8) on celite has been reported previously (11). Recovery of the virus was achieved by treatment with a concentrated solution of isinglass (1.2 to 6 per cent) which resulted in displacement of virus from the adsorbent.

TABLE IV

Elution of PVM from Celite

Mixture	Hemagglutination titer of supernate	Buffer		Hemagglutination titer of supernate from adsorbent resuspended in buffer
		Composition	pH	
PVM and saline..............	512	—	—	—
PVM and celite,* 0.5 gm........	64	$0.1 \text{ M NaH}_2\text{PO}_4 +$ Na_2HPO_4	7	0
" " " " "	64	" "	8	0
" " " " "	64	$0.1 \text{ M NH}_3 + \text{NH}_4\text{Cl}$	9	32
" " " " "	64	" "	10	64

* Celite analytical filter-aid.

It appeared of interest to determine whether adsorption and elution of influenza viruses could be achieved by procedures comparable to those employed with PVM.

The technique employed was almost identical to that described above. One-half gm. aliquots of the desired adsorbent were washed 3 times in buffered saline in the centrifuge and the final supernates decanted. To the packed adsorbent was added 2 cc. of influenza virus–infected allantoic fluid which, although not dialyzed, had been adjusted to pH 7 with N/10 HCl prior to use. The mixture was agitated for 10 minutes, the adsorbent sedimented, and the amount of unadsorbed virus in the supernate measured by the hemagglutination technique. The sediment was washed 3 times in buffered saline, and elution of the virus produced by the addition of 2 cc. of 0.1 M NH₃–NH₄Cl buffer at pH 10 to the packed adsorbent. The mixture was agitated for an additional 10 minutes, sedimented as before, and the supernate tested in the usual manner.

As is shown in Table V, adsorption of PR8 by all grades of celite tested was readily demonstrated. Elution of as much as 50 per cent of the adsorbed virus was obtained by the technique employed. It will be noted that the amount of virus which was eluted under these conditions varied considerably depending

upon the grade of celite used. In studies on the pH range over which PR8 was eluted from celite analytical filter-aid, by methods similar to those described above, it was found that with 0.1 M buffers of NH_3-NH_4Cl or glycine-NaOH at pH 9, no elution of virus was obtained, while at pH 10 approximately 25 per cent of the adsorbed virus was eluted. Serological identification of the hemagglutinating agent eluted from celite analytical filter-aid with glycine-NaOH buffer was carried out with specific anti-PR8 sera. It should be noted that PR8 did not elute from celite analytical filter-aid at pH 9, whereas PVM was eluted at this pH. This difference is probably attributable to differences in the viruses themselves. Adsorption of Lee virus and similar partial elution

TABLE V

Adsorption and Elution of Influenza Virus (PR8) with Various Adsorbents

Mixture PR8 and adsorbent		Hemagglutination titer of supernate	Hemagglutination titer of eluate[*]
	gm.		
Saline..	—	512	—
Celite 545...................................	0.5	32	256
Celite 503...................................	0.5	8	256
Hyflo-Super-cel.............................	0.5	0	64
Super-cel....................................	0.5	0	32
Celite 505...................................	0.5	4	128
Filter-cel....................................	0.5	0	32
Analytical filter-aid.........................	0.5	0	128

[*] 0.1 M NH_3-NH_4Cl buffer pH 10.

at pH 10 from celite analytical filter-aid also were demonstrated by the same procedure.

In the experiments described above, the recovery of PVM or of influenza viruses by elution at alkaline pH was considerably less than theoretical. Attempts were made to obtain the release of more virus by the successive addition of fresh alkaline buffer.

Experiments were carried out as described above employing 0.5 gm. aliquots of celite analytical filter-aid and 2 cc. of the desired virus suspension. After washing the virus-celite combination 3 times in buffered saline, virus was eluted in 2 cc. of 0.1 M NH_3-NH_4Cl buffer at pH 10 in the usual manner. The celite was sedimented, the alkaline supernate decanted, and the eluted virus in the supernate measured by the hemagglutination technique. An additional 2 cc. of alkaline buffer was then added to the packed adsorbent and the elution procedure repeated. This process was repeated a third time.

The results are recorded in Table VI. It will be seen that with PVM and the influenza viruses employed each additional aliquot of pH 10 buffer which was equilibrated with the celite adsorbent appeared to contain approximately 50

per cent as much eluted virus as the preceding aliquot. Within the limits of precision of the titration techniques used, these results suggest that the adsorbed viruses tended to be distributed between the solid phase and the liquid phase in relatively fixed proportions; of the order of 10 to 25 per cent of adsorbed virus was eluted at each successive step.

TABLE VI

Successive Elution of PVM and Influenza Viruses from Celite

Material tested	Dilution of supernate								
	4	8	16	32	64	128	256	512	1024
PVM and saline.............................	4*	4	3	3	3	3	3	2	0
Supernate after adsorption with 0.5 gm. celite‡ .	4	3	3	3	2	0			
Supernate after washing adsorbent.............	0	0	0	0					
First eluate, pH 10§.........................	4	3	3	3	2	0			
Second " " "...........................	4	3	3	2	0	0			
Third " " "...........................	3	2	0	0	0	0			
PR8 and saline.............................	4	3	3	3	3	3	3	2	0
Supernate after adsorption with 0.5 gm. celite ..	0	0	0	0					
Supernate after washing adsorbent.............	0	0	0	0					
First eluate, pH 10..........................	4	3	3	3	3	2	0		
Second " " "...........................	4	3	3	3	2	1	0		
Third " " "...........................	4	3	3	2	0	0	0		
Lee and saline..............................	4	4	4	3	3	3	3	2	0
Supernate after adsorption with 0.5 gm. celite ..	4	3	3	3	3	2	0		
Supernate after washing adsorbent.............	1	0	0	0					
First eluate, pH 10..........................	4	3	3	3	3	2	0		
Second " " "...........................	4	3	3	3	2	0	0		
Third " " "...........................	4	3	3	2	0	0	0		

* Degree of hemagglutination.
‡ Celite analytical filter-aid.
§ 0.1 M NH_3-NH_4Cl buffer.

DISCUSSION

The results of experiments described in this paper demonstrate that sedimentable particles derived from a wide variety of animal tissues possess the capacity to combine with PVM, though in varying degree. The virus can be dissociated in almost theoretical amount from combination with tissue particles of each sort merely by reducing the electrolyte concentration of the mixture, as was shown previously with mouse lung particles and RBC (6). Adsorption of PVM on and elution from various grades of celite also can be achieved by appropriate control of the ionic environment of the mixture. These findings

suggest that combination of PVM with tissue particles is the result of adsorptive forces operative at the surface of such particles, and may not be attributable to union in a chemical sense between the virus and some specific "receptor" substance present only in the lungs and RBC of susceptible species, as was postulated previously (2, 4, 5). That viruses may combine with one or more substances present in susceptible cells and that such cellular components may play an important rôle in virus synthesis or multiplication is a concept which is not challenged by the results obtained in this study. However, it seems apparent that the combination reaction between PVM and substances derived from tissues does not give indications of yielding important information as to the identity of the substance which may be essential for the multiplication of the virus.

The demonstration that both PVM and influenza viruses are adsorbed on and can be eluted from various adsorbents raises the possibility that under appropriately controlled conditions considerable purification of these agents could be achieved by taking advantage of the extraordinary properties of adsorbents in separative procedures. In preliminary experiments, not reported in detail in this paper, it has been found that not all the numerous substances present in a virus suspension were adsorbed under the conditions described in the experimental section. Moreover, only part of the substances which were adsorbed on celite were eluted at pH 9 or 10. The eluate, which in the case of influenza viruses contained between 25 and 50 per cent of the virus originally present, gave evidence of definite purification especially as regards reduction in non-virus protein. In other experiments in which either PVM or PR8 was adsorbed on celite packed in a column in a manner similar to that previously reported (12), elution of virus through the column was achieved with alkaline buffers. The extent to which either virus could be purified on a flowing chromatogram under practical conditions remains to be determined.

SUMMARY

Tissue particles from a wide variety of animal tissues possess the capacity to combine with PVM. Various adsorbents also combine with the virus. Elution of PVM from combination with either tissue particles or adsorbents can be achieved by appropriately altering the ionic environment. Influenza viruses also are adsorbed on and eluted from adsorbents under similar conditions. Reasons are presented for thinking that combination of PVM with tissue particles results from the action of adsorptive forces between virus and tissue particles.

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A CHEMICAL METHOD FOR THE DETECTION OF VIRUS INFECTION OF THE CHICK EMBRYO

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Chemical or physical differences in the allantoic fluid of normal and virus-infected chick embryos have been emphasized by few investigators. McLean et al.[1] mentioned differences in the pH of normal and influenza virus-infected allantoic fluids. Parodi and his collaborators[2] reported a slower decline in pH and an increase in volume of the allantoic fluid from embryos infected with influenza virus. In studies with their common cold virus (MR-1) Atlas and Hottle[3] noted high absorption peaks with dialyzed infected allantoic fluid which were attributed to protein. Such peaks were sometimes observed with normal fluid. In view of such observations, it appeared possible that there might be sufficient chemical difference in virus-infected and normal allantoic fluid to permit the development of a chemical test for virus infection of the chick embryo.

Early in the course of a systematic investigation of the properties of infected and normal allantoic fluid, it was discovered that allantoic fluid from embryos infected with influenza virus contained appreciably greater quantities of protein. Accordingly, a simple quantitative method for the determination of protein in allantoic fluid was devised and subsequently utilized in studies of infection of the allantoic sac with various viruses.

Materials and methods. Allantoic fluid. Allantoic fluids used in turbidity determinations were carefully harvested from 10–12-day-old White Leghorn embryos previously chilled for 12–18 hours at 4°C. Grossly bloody fluids and those inadvertently contaminated by yolk were discarded. Groups of 5–6 embryos were employed.

Viruses. The PR8 and Lee strains of influenza virus and a strain of Newcastle disease virus adapted to the allantoic sac by serial passage were used. The Habel strain of mumps virus, adapted in this laboratory to the allantoic sac, was also utilized. Semliki Forest virus (SFV) in the form of desiccated mouse brain (110th passage) was obtained through the courtesy of Dr. K. C.

[1] McLean, I. W., Jr., Cooper, G. K., Taylor, A. R., Beard, D., and Beard, J. W., Proc. Soc. Exp. Biol. and Med., 1945, 59, 192.

[2] Parodi, A. S., Lajmanovich, S., Pennimpede, F., and Mittelman, N., *J. Immunol.*, 1948, 58, 109.

[3] Atlas, L., and Hottle, G., *Science*, 1948, 108, 743.

Smithburn, who had demonstrated[4] rapid multiplication of the virus in the chick embryo. This mouse brain suspension killed embryos within 24–36 hours when injected into the allantoic sac. Subsequent passages were made with allantoic fluid.

Control materials. Allantoic fluids, hereinafter referred to as normal, were obtained from embryos inoculated with normal or heated (65°C for 30 minutes) allantoic fluid diluted 1:10 to 1:1,000 in 0.85% sodium chloride solution buffered to pH 7.2 with phosphate. The same diluent was used for virus inocula.

Turbidity determination. One cc of 10% trichloracetic acid is added to 1 cc of allantoic fluid in a soft glass test tube measuring 100 x 10 mm. Reagents are measured with ordinary serologic pipettes. Two to 5 minutes after the addition of acid, turbidity readings are determined in a Klett-Summerson colorimeter. An adapter for the small test tube and a blue filter (peak transmittance, 420 mμ) are employed. Mixing of reagents is accomplished by inversion of the colorimeter tube. A blank of 10% trichloracetic acid is used for preliminary setting of the zero reading of the colorimeter. In the instrument used in this study a 0.03% suspension of barium sulfate gives a reading of 70. Turbidity is expressed directly in the units comprising the scale of the colorimeter, these units being directly proportional to optical density. Fifteen serial determinations of turbidity, using the same allantoic fluid pool, showed an experimental error of ±2.8% for the procedure as described above.

Experimental. Studies of dialyzed allantoic fluids revealed a substance precipitable by 10% trichloracetic acid to be present in both normal and influenza virus-infected fluids and greatly increased in infected fluids. Similarly, micro-Kjeldahl determinations of total nitrogen demonstrated a higher concentration of nitrogen in fluid from infected embryos. The linear relation of the turbidity with trichloracetic acid and protein concentration of both normal and infected allantoic fluids may be seen in Fig. 1 in which the turbidities of varying dilutions of concentrated dialyzed allantoic fluids are plotted against the protein concentrations of the fluids as determined by the micro-Kjeldahl method. This relationship was found to hold with concentrations up to 500 turbidity units. However, specimens giving readings above 300 were diluted and re-examined because of difficulties experienced in obtaining accurate scale readings in the higher range. A similar straight line relationship between serum protein concentration and turbidity produced by trichloracetic acid has been established within certain limits of concentration by Chow *et al.*[5]

Further study of the acid-precipitable substance in allantoic fluid has indicated its protein nature. Dialysis in cellophane against 0.85% saline did not

[4] Smithburn, K. C., *J. Immunol.*, 1946, **52**, 309.

[5] Chow, B. F., Hall, L., Duffy, B. J., and Alper, C., *J. Lab. and Clin. Med.*, 1948, **33**, 1440.

reduce the concentration of the substance. The characteristic biuret, xantho-proteic, and ninhydrin color reactions were given by dialyzed allantoic fluids. Precipitates were formed in dialyzed and nondialyzed fluids by the addition of 95% ethyl alcohol or ammonium sulfate. The antigenicity of the substance, which is discussed below, is further evidence for its protein nature.
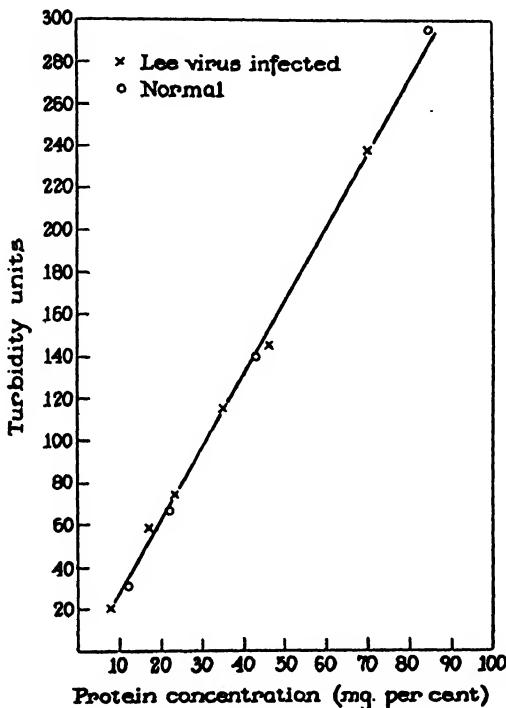
FIG. 1. Relation between turbidity and protein concentration in allantoic fluid. Turbidity was produced with 10% trichloracetic acid. Protein N₂ was determined by micro-Kjeldahl.

Fractionation of dialyzed allantoic fluids by half and full saturation with ammonium sulfate was performed. The protein concentration of these fractions was then determined by micro-Kjeldahl analysis after redialysis against saline. The albumin-globulin ratios in normal and infected fluids did not differ materially, being 7.3/1 in normal and 8/1 in infected fluids. The addition of trichloracetic acid to fractions obtained by ammonium sulfate precipitation produced turbidity equivalent to the protein nitrogen concentration. Ultraviolet absorption curves obtained with the Beckman spectrophotometer disclosed minima of 252 and 290 mμ and maxima of 265 mμ with both dialyzed normal and infected fluids of equal protein concentration (40 mg %). Absorption in this range is characteristic of proteins.

Electrophoretic studies* were made of dialyzed normal and infected allantoic fluids. Normal fluid was concentrated 23-fold, and infected fluid 9-fold, re-
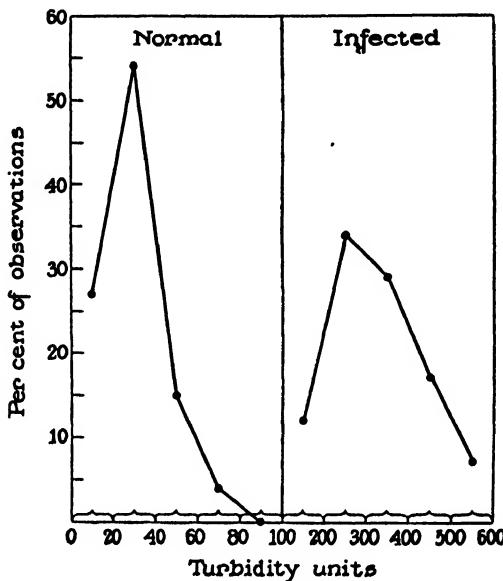
FIG. 2. Frequency distribution of turbidity values with normal and Lee virus infected allantoic fluids.

TABLE I

Increase in Allantoic Fluid Protein with Various Viruses

Virus 10⁻⁸ dilution inoculated	Time after noculation, days	No. of eggs	Allantoic fluid turbidity†	
			Range	Mean
Lee..........................	2	41	118–540	316
SFV*........................	1–1½	17	40–300	119
NDV........................	2	20	52–260	104
PR8.........................	2	12	30–152	87
MV.........................	4–5	12	56–160	84
Control.....................	2	100	8–75	29
"	4–5	10	21–50	33

    * 10⁻¹ to 10⁻⁸ dilutions used.
    † Turbidity developed with 10% trichloracetic acid.

sulting in protein concentrations of 375 and 300 mg %, respectively. These concentrations proved insufficient for accurate analysis of mobilities or sharp

    * Electrophoretic analysis was kindly carried out by Dr. Gertrude E. Perlmann, the Rockefeller Institute, New York City.

delineations of peaks; however, the rate of boundary migration and the degree of boundary spreading were consistent with what would be expected with protein solutions containing several components. With both normal and infected fluids at least 3 peaks were clearly discernible.

Comparison of normal and infected allantoic fluid protein concentrations. Studies of uninfected embryos demonstrated low concentrations of protein in the allantoic fluid as measured by turbidity produced with trichloracetic acid. In embryos of 10–12 days of age turbidity values varied from 8 to 75, representing protein concentrations of 2.4 to 23 mg % with an average value of 8.7 mg %. The frequency distribution of turbidity readings of allantoic fluids from 100 normal embryos is charted in Fig. 2 and compared with the range of turbidity (*i.e.* protein concentration) found in fluids from 41 embryos infected with the Lee strain of influenza virus. Turbidity readings of the infected allantoic fluids ranged from 118 to 540 (*c.f.* Table I), indicating concentrations of 35.4 to 162 mg % of protein.

Lee-infected allantoic fluid was subjected to differential centrifugation to determine to what extent sedimentable substances contributed to turbidity produced with trichloracetic acid. Low speed centrifugation (3,000 r.p.m. for 10 minutes) caused a 12% reduction in the turbidity observed initially, suggesting the presence of considerable cellular material. Total cell counts disclosed an average of 560 cells/cu mm in Lee-infected allantoic fluid contrasted with an average of 93 cells/cu mm in normal fluid. Further centrifugation at 37,900 g for 30 minutes resulted in a further reduction of the turbidity produced with acid amounting to 8% of the turbidity originally present. Thus, Lee virus, itself, contributes little, if any, to the turbidity of infected allantoic fluid, as this amount of centrifugation leaves less than 1% of the virus in the supernate.

Source of protein in infected allantoic fluid. It appeared likely that the increased protein in the allantoic fluid of virus-infected embryos was attributable to host reaction and perhaps to destruction of host tissue, especially in view of the increased number of cells in infected fluid. Evidence cited above demonstrated that the virus itself did not contribute to the turbidity produced with acid, and further studies have shown no direct relation between virus and protein concentrations. Moreover, experiments with Lee virus demonstrated a difference in the rate of virus multiplication and the increase in allantoic fluid turbidity as is shown graphically in Fig. 3. Corroboration of this difference was obtained in experiments in which the time required to reach maximal virus concentration was varied by the use of inocula of differing dilutions of virus. The results are presented in Table II.

The protein of infected allantoic fluid did not differ immunologically from the protein normally present in allantoic fluid. Sera from rabbits injected intravenously with dialyzed infected or normal allantoic fluid contained antibodies capable of forming precipitates with concentrated allantoic fluid from

either infected or normal embryos. These antibodies were absorbed from either antiserum by normal or infected allantoic fluid antigen, as well as by a suspension of normal chorio-allantoic membrane (C.A.M.). These data are summarized in Table III.

Viruses which cause an increase in allantoic fluid protein. The Lee and PR8 strains of influenza virus, Semliki Forest virus, Newcastle disease virus, and mumps virus consistently caused an increase in allantoic fluid protein during the course of infection of the allantoic sac. A correlation may be drawn between the toxicity of the viruses studied and the degree of protein increase. Lee and Newcastle disease viruses, which killed embryos after 48 hours, and Semliki
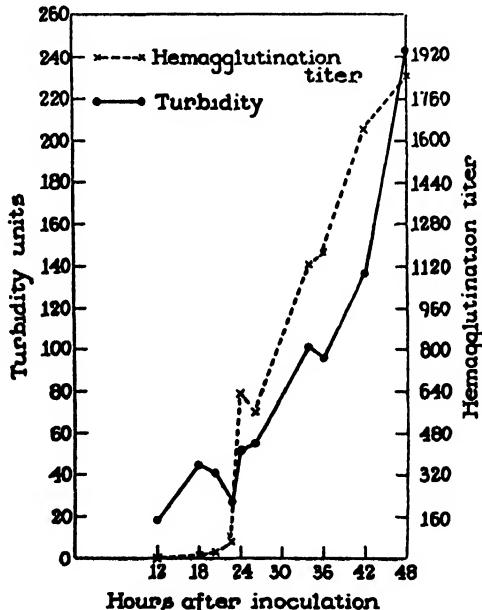
FIG. 3. Temporal relation between increase in turbidity and Lee virus concentration in allantoic fluid.

Forest virus, which killed even sooner, caused significantly more turbidity in allantoic fluid than did the considerably less toxic PR8 and mumps strains, as is shown in Table I.

Nonviral causes of protein increase in allantoic fluid. The production of the turbidity reaction by 5 different viruses is evidence of its non-specificity. It is obvious that any reaction dependent upon response or destruction of host tissue may be induced by chemical or physical agents as well as infectious ones. Thus, it was found that injection of 0.1 cc quantities of broth or serum might increase the allantoic fluid protein of embryos beyond the low concentrations usually seen. Such increases were greater than would be anticipated on the

TABLE II

Relation of Increase in Allantoic Fluid Turbidity to Extent of Multiplication of Lee Virus

Virus dilution inoculated	Time after inoculation					
	24 hr		36 hr		48 hr	
	Hem. titer[*]	Turb.[†]	Hem. titer	Turb.	Hem. titer	Turb.
10^{-3}	1:636	53	1:1331	96	1:1843	243
10^{-5}	0	28	1:1536	93	1:2048	218
10^{-7}	0	21	1:65	42	1:1229	145

* Mean hemagglutination titer of allantoic fluids.

† Mean turbidity value of allantoic fluids.

TABLE III

Absorption Experiments with Antisera Against Normal and Infected Allantoic Fluid Protein

Rabbit serum		Precipitin titer[*]	
	Absorbed with	Normal all. fl. prot.	Infected all. fl. prot.
Anti normal all. fl. prot..............	—	1:250	1:250
" " " " "	Infected all. fl. prot.	0	0
Anti infected all. fl. prot..............	—	1:250	1:250
" " " " "	Normal all. fl. prot.	0	0
" " " " "	" C.A.M.	0	0

* Highest dilution of antigen which gave a positive reaction with serum diluted 1:2.

TABLE IV

Prevention of Protein Increase in Allantoic Fluid by Virus Antiserum

Inoculum		Mean hemagglutination titer of allantoic fluids	Mean turbidity value of allantoic fluids
Serum	Lee virus dilution		
—	10^{-6}	1:1877	150
Normal 1:100......................	"	1:2046	93
" 1:500......................	"	1:2253	188
Anti Lee 1:100......................	"	0	25
" " 1:500......................	"	1:2	19
" " 1:100......................	—	.—	20
" " 1:500......................	—	—	42

basis of the amount of protein injected, demonstrating an actual reaction of the embryonic tissue to the material introduced.

When broth was injected in control embryos, mean turbidity values were almost double (*i.e.* 48 units) those observed in saline inoculated embryos, and occasional fluids exceeded 100 turbidity units. The injection of undiluted allantoic fluid occasioned similar non-specific response, although it was of lesser degree (mean turbidity, 38 units), and only 10% of individual fluids exceeded the upper limit of 75 turbidity units observed in normal embryos (Table I).

The effect of bacterial infection of the chick embryo was not systematically studied, but examination of bacterially contaminated allantoic fluids disclosed increased turbidity, *i.e.* more than 75 units, in only 2 of 16 instances. In any event, such fluids are unsuitable for virus study, and are customarily discarded.

In normal embryos incubated for more than 12 days, sharp, capricious increases in the allantoic fluid protein may occur, making such embryos unsuitable for use with the present method. Fluids grossly contaminated by blood introduced at the time of harvest also proved useless because of the presence of extraneous serum protein.

Prevention of protein increase by virus antiserum. Neutralization of Lee virus by specific immune rabbit serum prevented the increase in allantoic fluid protein which occurred following the injection of virus and normal serum or virus alone. The results are summarized in Table IV. It will be seen that, in the dilutions indicated, inactivated rabbit serum *per se* caused no undue elevation of mean turbidity. In other experiments dilutions of rabbit serum as low as 1:40 were used without increase in allantoic fluid protein. This experiment affords definitive evidence that the protein increase which follows introduction of virus into the allantoic sac is a corollary of virus multiplication.

Discussion. The study of many animal viruses and attempts to recover new viruses have been handicapped by the lack of simple *in vitro* technics comparable to the hemagglutination reaction. The method outlined in the present communication has the virtue of simplicity, and in theory may be of value in the detection of any virus capable of multiplication in the cells of the allantoic sac of the chick embryo. Because the method is dependent upon the vagaries of host response, it is unsuitable for the direct measurement of virus concentration. However, both specificity and quantitation may be obtained by the employment of immune serum. It is conceivable that a virus might be recovered and identified immunologically as the etiological agent of a disease solely by chemical evidence of infection of the chick embryo.

Summary. A method is described for the detection of virus infection of the allantoic sac of the chick embryo. The method is dependent upon the increased concentration of protein in infected allantoic fluid. Protein concentration is measured by determining the degree of turbidity produced upon the addition of 10% trichloracetic acid to allantoic fluid.

While this article was in press, Polson and Dent (*Nature*, 1949, **164**, 233) described increases in the protein concentration of allantoic fluid from eggs infected with lumpy skin disease virus or blue tongue virus.

HEMAGGLUTINATION WITH THE GDVII STRAIN OF MOUSE ENCEPHALOMYELITIS VIRUS

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The capacity of certain viruses to cause agglutination of erythrocytes[1] has permitted the development of *in vitro* procedures which have greatly facilitated investigative and diagnostic work with these agents. Although at least 10 different animal viruses are known to cause hemagglutination (pertinent data have been summarized recently),[2] there is almost no evidence indicating that any of the neurotropic viruses possesses a similar capacity. Recently, however, Bremer and Mutsaars[3] stated that the Lansing strain of poliomyelitis virus caused agglutination of sheep RBC, and Hallauer[4] stated that Columbia SK and Columbia MM viruses also agglutinated sheep RBC. We have been unable to confirm the results reported for the Lansing strain, as too have other workers.[5] However, in the accompanying paper Olitsky and Yager[5] have confirmed and extended the results reported for SK and MM viruses.

The present study was concerned chiefly with the GDVII strain of mouse encephalomyelitis virus as well as with the FA strain.[6] In addition, the Lansing, MEF1, and Brunhilde strains of poliomyelitis virus were investigated. It will be demonstrated that the GDVII strain causes agglutination of human RBC at 4°C, but not at 23 or 37°C; that such hemagglutination is inhibited by homologous immune serum, and by anti-FA virus serum, but not by antiserum against other viruses. No evidence of hemagglutination could be obtained with the FA strain nor with any of the strains of poliomyelitis virus which were employed.

Materials and methods. Viruses. The GDVII strain was obtained from Dr. Max Theiler, I.H.D. Laboratories, The Rockefeller Foundation, New York City. Three FA strains were used; one obtained from Dr. Theiler and 2 obtained from Dr. J. Melnick, Yale University, New Haven, Conn. Three poliomyelitis virus strains were employed; the Brunhilde strain was kindly supplied by Dr.

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[2] Smadel, J. E., Viral and Rickettsial Infections of Man, 1948, chap. 3, J. B. Lippincott Co., Philadelphia, Pa.
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[4] Hallauer, C., 4th Internat. Cong. Microbiol., July 20–26, 1947, Copenhagen, 1949, p. 257.
[5] Olitsky, P. K., and Yager, R. H., accompanying paper.
[6] Theiler, M., and Gard, S., *J. Exp. Med.*, 1940, **72**, 40.

D. Bodian, The Johns Hopkins University, Baltimore, Md., and the Lansing and MEF1 strains were obtained from Dr. P. K. Olitsky, The Rockefeller Institute, New York City. The Brunhilde strain was contained in infected monkey spinal cord. The other viruses were maintained by occasional intracerebral passage in mice. Brains were removed from exsanguinated mice shortly after the appearance of signs indicating infection of the central nervous system. Ten per cent brain suspensions were prepared with 0.01 M phosphate buffer at pH 7.2. The suspensions were ground for $2\frac{1}{2}$ minutes in a modified Waring Blendor which was cooled with ice and then were centrifuged for 15 minutes at 7,760 g. The supernates were employed either promptly after preparation or following storage at $-70°C$, sometimes for as long as 14 days. Virus titrations were performed by the intracerebral technic using serial 10-fold dilutions in 10% normal rabbit serum saline. A group of 5 or 6 mice was used for each dilution and the 50% infectivity end point, LD_{50}, was calculated in the usual manner.

Hemagglutination technic. Hemagglutination titrations were carried out in a manner similar to that employed with influenza virus.[1] Serial 2-fold dilutions of brain suspensions in saline buffered at pH 7.2 and a final concentration of 0.25% human Group O erythrocytes were employed. With the GDVII strain the mixtures were held at 4°C for 2 hours. Readings were recorded in the usual manner and the end point was taken as the highest dilution which gave a 2+ reaction.

Hemagglutination-inhibition technic. Antibody titrations were carried out with serial 2-fold dilutions of inactivated (56°C/30 min.) sera in buffered saline and a constant amount of virus, usually 16 hemagglutinating units. A final concentration of 0.25% RBC was used and readings were made after 2 hours at 4°C. The end point was taken as the highest dilution of serum which completely inhibited hemagglutination.

Immune sera. Through the courtesy of Dr. P.K. Olitsky immune sera against a large number of different neurotropic viruses were made available. In most instances the sera were obtained from rabbits which had been repeatedly injected intraperitoneally or subcutaneously with infected mouse brain. In some instances sera were also obtained from immunized guinea pigs, mice or monkeys. Immune sera and control normal sera usually were stored at $-30°C$.

Hemagglutination with GDVII virus. Positive results were obtained in hemagglutination experiments with the GDVII strain when (a) suspensions of infected mouse brain, (b) human Group O erythrocytes, and (c) a reaction temperature of 4°C were employed. The results of typical experiments are shown in Table I. High titers ranging from 1:2,000 to 1:16,000 or more were commonly obtained with 0.25% RBC. In general, the hemagglutination titer was inversely proportional to the concentration of RBC, as is the case also with influenza virus.[7]

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[7] Whitman, L., *J. Immunol.*, 1947, **56**, 167.

Hemagglutination occurred only if the mixtures were cold (4°C), disappeared rapidly when cold mixtures were warmed either at room temperature (23°C) or at 37°C, and reappeared when the mixtures were again cooled to 4°C. The reaction developed relatively slowly and, although clear evidence of hemagglutination was present at one hour, more definite agglutination was present at 2 hours. The pattern of agglutinated cells was closely similar to that observed with human RBC and either influenza or mumps virus.

TABLE I

Hemagglutination with GDVII Strain of Mouse Encephalomyelitis Virus

Supernate of mouse brain suspension	Human group O erythrocytes, %	Held 2 hr at °C	Final dilution of supernate									
			125	250	500	1000	2000	4000	8000	16,000	32,000	64,000
GDVII strain	0.25	4	4*	4	4	4	4	4	3	1	±	0
" "	0.25	23	0	0	0	0	0					
" "	0.25	37	0	0	0	0	0					
" "	2.5	4	4	4	2	0	0	0	0	0	0	0
" "	1.0	4	4	4	4	3	2	1	0	0	0	0
" "	0.5	4	4	4	4	4	3	2	1	±	0	0
" "	0.25	4	4	4	4	4	4	4	3	1	±	0
FA "	0.25	4	0	0	0	0	0					
Poliomyelitis, Lansing	0.25	4	0	0	0	0	0					
" MEF1	0.25	4	0	0	0	0	0					
Normal, control..........	0.25	4	0	0	0	0	0					

* Indicates degree of hemagglutination.

Despite numerous attempts employing a wide range of experimental conditions, it was not possible to obtain evidence of hemagglutination with the FA strain nor with the Lansing, MEF1 or Brunhilde strains of poliomyelitis virus. In addition to human RBC, erythrocytes from the following species were used: monkey, horse, sheep, cat, dog, guinea pig, hamster, mouse and chicken. The GDVII strain was incapable of causing agglutination of any RBC other than those derived from man. Supernates of normal mouse brain suspensions, prepared as described above, did not cause agglutination of human RBC at dilutions greater than 1:4. As is pointed out also in the accompanying paper,[5] erythrocytes of certain species, *e.g.*, hamster, dog, cat and guinea pig, as well as the mouse, commonly showed agglutination when mixed with normal mouse brain suspensions.

The agglutination of human RBC which is caused by GDVII virus in the cold disappears after a few minutes at room temperature. Because of this, both the reaction and readings of titrations are best carried out in the cold room. As is shown below, agglutination is associated with adsorption of the virus to RBC and the dispersal of the agglutinated cells is associated with elution of the

virus from them. Successive cycles of adsorption and elution, dependent merely on changes in temperature, can be repeated at will with a single mixture of RBC and GDVII virus. Four such cycles have been carried out.

Hemagglutination-inhibition with immune serum. Agglutination of human RBC with GDVII virus in the cold was prevented by high dilutions of anti-GDVII serum as well as anti-FA serum but not by immune serum against other viruses. The results of typical experiments are shown in Table II. Hemagglutination-inhibition titers ranging from 1:4,000 to 1:16,000 were obtained commonly with anti-GDVII serum and similar high titers were obtained also with anti-FA serum. Normal serum usually caused some non-specific inhibition; with rabbit and guinea pig serum titers of 1:32 or lower were commonly found;

TABLE II

Inhibition of Hemagglutination with GDVII Virus by Immune Serum

Serum		GDVII virus units	Held 2 hr at °C	Serum hemagglut. inhibition titer
Immune vs.	Species			
Normal m.br*........................	Rabbit	16	4	0†
GDVII m.br...........................	"	16	4	8000
Normal m.br..........................	Mouse	16	4	0
FA m.br..............................	"	16	4	8000
" "	Monkey	16	4	8000
Poliomyelitis (conval.).................	"	16	4	0
" Lansing m.br..............	Rabbit	16	4	0
Mengo, m.br.........................	"	16	4	0

* m.br. = Mouse brain.
† 0 = No inhibition of hemagglutination at 1:32 serum dilution.

with human and mouse serum titers as high as 1:128 were encountered. In some instances heating at 56°C for 30 minutes reduced the degree of non-specific inhibition. It should be emphasized that the injection of mouse-brain suspensions into animals other than mice commonly results in the development of antibodies which cause agglutination of human RBC. Usually the agglutination titer of such sera is not greater than 1:128 but in occasional instances it may be considerably higher. With immune serum agglutination of RBC occurs not only at 4°C but also at room temperature or at 37°C, as not with GDVII virus. The agglutinins were readily removed from such sera by absorption with 20% human RBC at 4°C. Absorbed immune sera gave hemagglutination-inhibition titers with GDVII virus which were identical with those obtained with unabsorbed sera.

Immune sera against the following viruses were employed in hemagglutination-inhibition experiments with GDVII virus: lymphocytic choriomeningitis, Eastern equine encephalitis, Japanese B encephalitis, St. Louis encephalitis,

Russian Far East encephalitis, vesicular stomatitis, West Nile, rabies, herpes simplex, vaccinia, Columbia SK, Columbia MM, Mengo encephalomyelitis, encephalomyocarditis, influenza A (PR8 strain) and PVM. In no instance was significant inhibition of GDVII virus demonstrable with these sera.

In cross-immunity experiments Theiler and Gard[6] demonstrated an immunological relationship between GDVII and FA viruses. It appears of considerable interest that the results of hemagglutination-inhibition experiments indicate not only that antibody specifically directed against GDVII virus is present in high titer in immune sera but also show clear evidence of a close antigenic relationship to FA virus.
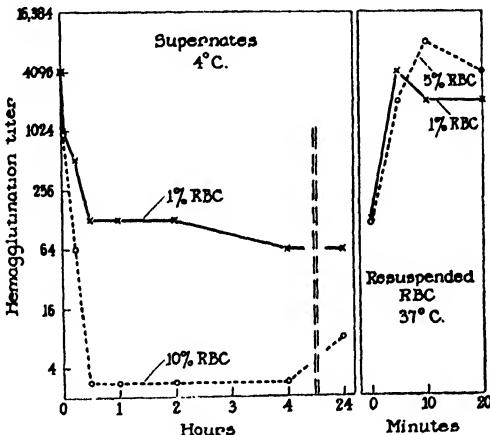
FIG. 1. Adsorption of GDVII virus on human Group O RBC at 4°C and elution at 37°C. Hemagglutination titer of supernates from mixtures of virus and RBC is plotted against time; mixtures were held at 4°C. Hemagglutination titer of supernates from resuspended RBC is plotted against time; such erythrocytes were held at 37°C

Adsorption and elution of GDVII virus. When mixtures of human RBC and GDVII virus were held at 4°C, the virus was adsorbed rapidly by the erythrocytes and sedimented with them on light centrifugation. When the sedimented RBC were resuspended in buffered saline and held at 4°C, elution of the virus did not occur. However, when the resuspended RBC were warmed to 37°C, elution of the virus occurred very rapidly and maximum titers were obtained in the supernate within 5 to 10 minutes. Results of typical experiments are shown in Fig. 1. As would be expected, the concentrations of RBC employed affected the extent to which the virus was adsorbed at 4°C but did not have any striking effect on the rate or degree of elution at 37°C. Mixtures held at 4°C for as long as 24 hours showed no significant elution of the virus from RBC.

That hemagglutination with GDVII virus is caused by the virus particle itself and not by a component separable from the virus is indicated by the results shown in Table III.

Adsorption of a suspension with human RBC at 4°C resulted in reductions in the hemagglutination and virus infectivity titers of the supernate which were of similar degree. Moreover, on warming the resuspended RBC at 37°C, similar increases in both titers occurred indicating that elution of the virus was effected at the higher temperature.

Properties of hemagglutination component. The hemagglutination titer of GDVII mouse brain suspension was not diminished by storage at 4°C for 43 days. Heating crude suspensions in saline at 56°C for 30 minutes caused marked loss, *i.e.* 99%, of hemagglutinating capacity. On the other hand, suspensions prepared from brain material extracted by methanol in the cold showed only a 2- to 4-fold reduction in titer on similar heating. Moreover, such suspensions showed hemagglutination titers of 1:1,000 after heating at 65°C for 30 minutes. Centrifugation at 7,760 g for 30 minutes did not reduce the hemagglutination

TABLE III

Adsorption on and Elution from Human RBC of GDVII Virus

Material tested	Hemagglutination titer* at 4°C vs. human RBC	Virus infectivity titer* in mice I.C., LD_{50}
GDVII m.br. suspension	10,240	$10^{-8.1}$
Supernate after adsorption with 10% RBC, 1 hr at 4°C	160	$10^{-5.6}$
Supernate of resuspended RBC in saline, 15 min. at 37°C	10,240	$10^{-7.8}$

* Titers are expressed in terms of final dilution of brain material.

titer of suspensions. Filtration through Seitz-EK pads caused an 8-fold reduction in the titer of the filtrate. Suspensions buffered at pH values from 4.8 to 8.3 gave similar titers. The amount of virus adsorbed by human RBC at 4°C was not significantly affected by the pH of the mixture within this range. Furthermore, elution of virus from RBC at 37°C was complete when erythrocytes were resuspended in buffer of pH 4.8 to 8.

Concentration of GDVII virus. By resuspension of RBC with adsorbed virus in small volumes of buffered saline and warming the suspension to 37°C, it was possible to achieve considerable concentration (10 times or more) of the virus in the eluate. In most instances the increase in titer obtained was as great as or greater than would have been expected in terms of the volumes of eluate employed. Either high or low titer suspensions as well as suspensions which had been diluted before adsorption yielded satisfactory results in concentration experiments of this kind.

Recently it was reported[8] that GDVII virus could be purified considerably by precipitation with 25 to 30% methanol in the cold. In the present study numer-

[8] Brumfield, H. P., Stulberg, C. S., and Halvorson, H. O., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 410.

ous attempts were made to concentrate the virus by means of such a procedure. In all instances the virus titer was determined by the hemagglutination technic. It was found that 52% methanol mixtures held at 4°C for 3 hours yielded better results than mixtures at other methanol concentrations. Despite the use of a large variety of experimental conditions which included variations in pH, ionic strength, amount of centrifugation before and after the addition of methanol, as well as extraction of brain material with organic solvents, it was not possible to obtain consistent results. In some experiments 10-fold or greater concentration was achieved but the results were not sufficiently reproducible to make the procedure valuable. Moreover, after concentration by methanol precipitation, the virus appeared to be unstable and hemagglutination titers decreased rapidly on storage of concentrated material at 4°C.

Failure of FA virus to cause hemagglutination. The infectivity titer of GDVII virus is definitely higher than that of FA virus. With the strains employed in this study, GDVII gave LD_{50} titers of the order of 10^{-8} or more while FA gave titers of the order of 10^{-6} or less. On the assumption that the infectivity titer is proportional to the virus concentration, it seemed possible that the failure to demonstrate hemagglutination with FA might be attributable to a relatively low concentration of the agent in infected brain tissue. Because of the numerous similar properties of the 2 viruses[6] and the close antigenic relationship disclosed in the hemagglutination-inhibition experiments described above, it appeared desirable to determine if FA shared with GDVII the capacity to cause hemagglutination of human RBC in the cold.

Attempts to concentrate FA virus, as was feasible with GDVII, by adsorption on human RBC at 4°C and elution in a small volume of diluent at 37°C, were uniformly unsuccessful. In no instance was hemagglutination demonstrable with the eluates despite the use of erythrocytes derived from numerous species. Moreover, the results of infectivity titrations indicated that FA virus was not adsorbed by human RBC under the conditions employed; supernates of virus-RBC mixtures held at 4°C showed no reduction in infectivity titer. It appears, therefore, that despite similarities relative to numerous properties FA virus and GDVII virus do not give similar reactions with human erythrocytes *in vitro.*

Discussion. That certain neurotropic viruses possess the capacity to agglutinate in the cold erythrocytes deriving from certain animal species appears evident from the results of this study and that described in the accompanying paper.[5] By means of the hemagglutination reaction with human RBC at 4°C it is possible to estimate *in vitro* the concentration of GDVII virus in a suspension of infected mouse brain. As is the case with other animal viruses which cause hemagglutination, relatively high concentrations are required before positive results are obtained; with GDVII of the order of 10^4 mouse infectious doses of virus correspond to one hemagglutinating unit. The available evidence suggests

that the infective virus particle is itself responsible for hemagglutination with this agent. By means of the hemagglutination-inhibition technic, also carried out with human RBC at 4°C, the concentration of antibodies in immune serum specifically directed against the virus can be estimated *in vitro*. Evidence obtained in hemagglutination-inhibition experiments indicates that GDVII virus is immunologically closely related to FA virus, but is not related to any other of the numerous agents tested.

Despite numerous attempts with a wide variety of experimental conditions, it was not possible to demonstrate hemagglutination with FA virus. Moreover, with the Lansing, MEF1 and Brunhilde strains of poliomyelitis virus no evidence was obtained indicative of a capacity to combine with erythrocytes. It may be pertinent that neither FA virus nor poliomyelitis virus reaches high titers in infected central nervous system tissue and it is possible that the failure to show hemagglutination with these agents is attributable to insufficient concentration. On the other hand, qualitative factors also may be of critical importance and it seems possible that with erythrocytes from still other species and with different experimental conditions positive results might be obtained.

The dependence of hemagglutination with GDVII virus and of adsorption of the agent by human RBC upon a low temperature, *i.e.* 4°C, appears to be unique. With the exception of the neurotropic viruses discussed in the accompanying paper,[5] other viruses which cause hemagglutination show no such temperature effect.

Summary. Suspensions of mouse brain infected with the GDVII strain of mouse encephalomyelitis virus cause agglutination of human Group O RBC at 4°C. Anti-GDVII virus serum inhibits hemagglutination by the agent as also does anti-FA virus serum. GDVII virus is adsorbed by human RBC at 4°C and rapidly elutes from them at 37°C. Three strains of poliomyelitis virus failed to show any evidence of hemagglutination.

A RESISTANT VARIANT OF MUMPS VIRUS

Multiplication of the Variant in the Presence of Inhibitory Quantities of Friedländer Bacillus Polysaccharide

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Evidence obtained in previous studies (1–3) on the inhibition of multiplication of mumps virus by the capsular polysaccharides of Friedländer bacilli raised the possibility that the individual viral particles are not all entirely identical and suggested that some might differ from others as to mechanism of multiplication. The following findings are pertinent: When small quantities of virus (*i.e.*, 10^2 E.I.D.) were inoculated, the injection of polysaccharide caused quantitative inhibition in multiplication which was reflected in the production of only 0.5 to 10 per cent of the maximum concentration of virus but did not prevent completely the multiplication of the agent. Even when relatively large amounts of polysaccharide (*i.e.*, 1.0 mg. per embryo) were injected, slight multiplication of the virus occurred although the degree of inhibition was striking. When large quantities of virus (*i.e.*, 10^5 E.I.D.) were given, the injection of polysaccharide did not result in any significant inhibition of multiplication (2). The range of the variables studied was sufficiently wide, and the results reproducible enough to make it appear improbable that quantitative factors alone were to be held responsible for the findings. More probable seemed the possibility that qualitative variables were operative, and the idea arose that mumps virus preparations contained, in addition to the predominant typical viral particles, a numerically small proportion of variant particles which, unlike the typical virus, were capable of unrestricted multiplication in the presence of the polysaccharide. It seemed likely that, should such variant viral particles be present, they could be revealed readily because of the availability of a chemical agent which would preferentially select them from the viral populations under study.

The hypothesis was subjected to experimental test. The results obtained appear to indicate clearly that a variant is present in mumps virus preparations; that the variant is capable of unrestricted multiplication in the presence of large amounts of polysaccharide; that the variant is, as to other properties tested, indistinguishable from the original strain; that the only means at hand for demonstrating the presence of the variant is polysaccharide which is active as an inhibitor of mumps virus multiplication.

Materials and Methods

Viruses.—The following viruses were employed: mumps (Habel strain); influenza A, PR8 strain; influenza B, Lee strain. For convenience, they will be referred to hereafter as mumps, PR8, and Lee. They were cultivated in the allantoic sac of chick embryos which were 7 to 9 days of age for mumps and 9 to 11 days of age for PR8 or Lee. The methods of cultivation and the procedures for identification were identical to those previously described (3). Between passages each of the viruses was stored in a solid carbon dioxide cabinet at $-70°C$. In certain instances mumps virus during serial passages was stored for short periods in an electrically operated refrigerator at $-30°C$. As reported recently (4) the virus can be maintained without reduction in infectivity for at least 3 months at the latter temperature.

Virus Infectivity and Hemagglutination Titrations.—The methods employed were identical to those described in earlier reports from this laboratory (2). Infectivity titrations were carried out with serial tenfold dilutions which were inoculated into the allantoic sac of chick embryos. Hemagglutination titrations were carried out with serial twofold dilutions which were mixed with an equal volume of 1.0 per cent chicken RBC.

Polysaccharide Preparations.—The capsular polysaccharides of Friedländer bacillus type A (Fr.A), type B (Fr.B), and type C (Fr.C), respectively, (5, 6) were employed.[1] Polysaccharide solutions were prepared in 0.85 per cent sodium chloride solution buffered at pH 7.2 (0.05 M phosphate). Each solution was heated at $70°C.$ for 30 minutes before use.

Immune Serum.—Rabbits were injected intravenously with 10 cc. of undiluted infected allantoic fluid, and this was followed at 2 week intervals by two intraperitoneal injections of 10 cc. of similarly infected fluid. Two weeks after the last injection blood was withdrawn, the serum separated, and stored at $4°C$. The immune serum was absorbed with normal embryo tissue and inactivated by heating at $65°C.$ as previously described (3).

Reproducibility of End Points.—In an earlier paper (2) the reproducibility of hemagglutination titration end points with mumps virus was determined in 33 groups of control infected embryos. In the present study similar computations were carried out with end points obtained in an additional series of 38 groups of control infected embryos. The standard deviation of the distribution of end points was 0.331 log unit (2.1-fold), a value slightly lower than that previously obtained. Thus, a difference of 0.94 log unit (8.7-fold) between the end points found in any two groups should occur by chance only once in 20 times (7). As in previous studies (1, 2) the arithmetic mean of the individual allantoic fluid end points was employed in the present investigation. Comparative computations with the available data indicate that the use of the geometric mean does not yield any statistical advantage, and does not significantly alter the probability values.

EXPERIMENTAL

Demonstration of a Variant of Mumps Virus by Serial Passage in the Presence of the Capsular Polysaccharide of Friedländer Bacillus Type B.—Earlier studies (1, 2) revealed that, despite the marked inhibition of multiplication of mumps virus which was caused by the capsular polysaccharides of Friedländer bacilli, multiplication was not prevented completely. It was also demonstrated that when very large inocula of virus were employed no significant inhibition of multiplication was demonstrable. As an explanation of these findings, it seemed probable that the multiplication observed might be initiated by viral particles

[1] The polysaccharides were obtained through the kindness of Dr. Walther F. Goebel, The Rockefeller Institute for Medical Research.

which differed from the predominant particles comprising the viral population under study. In an attempt to demonstrate the presence of a variant, resistant to the inhibitory activity of Friedländer type B polysaccharide (Fr. B), mumps virus was subjected to serial passage in the allantoic sac in the presence of small but inhibitory quantities of Fr.B. After each successive passage the capacity of the strain to multiply in the presence of Fr.B was determined.

Approximately 10^2 embryo infectious doses (E.I.D.) of mumps virus was inoculated intra-allantoically into two groups of 9 day chick embryos. After 1 hour one group was given Fr.B intra-allantoically, 0.1 mg. per embryo, and the embryos of the other group received 0.1 cc. of saline. After incubation at 35°C. for 6 days, the eggs were chilled at 4°C. overnight, the allantoic fluids removed, and their hemagglutination titers determined. The allantoic fluid with the highest titer from the group which had received Fr.B was used as inoculum for the next serial passage. It was diluted 10^{-5} in 10 per cent normal horse serum broth, and inoculated intra-allantoically in groups of embryos which were treated as above. From the control groups an allantoic fluid was selected which had a hemagglutination titer similar to that of the fluid selected from the polysaccharide-treated group. This fluid, diluted 10^{-5}, was used as inoculum for the next serial passage in the control line. In subsequent passages 0.05 mg. of Fr.B per embryo was employed. After serial passage 1.0 mg. of Fr.B per embryo was employed to determine the degree of polysaccharide resistance of the two passage strains. In most instances during serial passage 500 units of crystalline penicillin G and 5 mg. of streptomycin per embryo were injected along with the inoculum in order to insure sterility.

The results of significant portions of one such experiment are presented in Table I. It will be noted that following but two passages of mumps virus in the presence of relatively small quantities of polysaccharide, the multiplication of the virus was not significantly inhibited by Fr.B. After 10 passages in the presence of Fr.B quantities of the carbohydrate as large as 1.0 mg. per embryo did not prevent this strain from multiplying in an unaltered manner. In addition, it was found that equally large quantities of capsular polysaccharide from type A or type C Friedländer bacilli did not diminish the multiplication of this resistant strain. As is evident, marked inhibition of multiplication was demonstrable at every step in the control series with mumps virus which was passed in parallel, but in the absence of the polysaccharide.

In another experiment carried out in an identical manner, 10 passages in the presence of Fr.B were required before a stable resistant strain of mumps virus was obtained. The initial virus suspension which was used in the latter experiment was different from that employed in the experiment described above.

As is indicated below, resistant variants, once obtained, retained their capacity to multiply in unrestricted manner in the presence of polysaccharide after as many as 3 to 4 serial passages in the absence of the substance.

Demonstration of a Resistant Variant of Mumps Virus after a Single Passage in the Presence of Fr.B.—When it was found that a resistant variant could be obtained at will upon serial passage of mumps virus in the presence of Fr.B, attempts were made to obtain similar variants in a single passage. If, as seemed probable, the variant virus was present in a low concentration in suspensions

TABLE I

Demonstration of a Resistant Variant of Mumps Virus by Serial Passage in the Presence of the Capsular Polysaccharide of Friedländer Bacillus, Typ β

Serial passage of mumps virus			Mean hemagglutination titer* of allantoic fluids‡ of embryos inoculated with virus and injected with			Difference in titers of NaCl and Fr.B groups	
In presence of	No. of passages	Dilution employed	NaCl§	Fr.B§		fold	log
mg./embryo				mg./embryo		fold	log
—	—	Parent strain	10⁻⁵	336	0.10	16	−21×	−1.33
Fr.B	0.10	1	"	597	0.05	48	−12×	−1.10
"	0.05	2	"	264	"	232	0	−0.05
"	"	4	"	853	"	544	"	−0.19
"	"	10	"	240	"	299	"	+0.10
"	"	"	"	"	1.00	213	"	−0.05
NaCl		1	"	64	0.05	3	−23×	−1.36
"		2	"	256	"	24	−12×	−1.10
"		4	"	469	"	42	−11×	−1.06
"		10	"	95	"	12	− 8×	−0.90
"		"	"	"	1.00	0	−95×	−1.98

* Expressed as the reciprocal of the hemagglutination titer.
‡ Allantoic fluids were harvested 6 days after inoculation.
§ Injection of NaCl or Fr.B was given 1 hour after the virus.

TABLE II

Demonstration of a Resistant Variant of Mumps Virus in a Single Passage in the Presence of the Capsular Polysaccharide of Friedländer Bacillus, Type B

Serial passage of mumps virus			Mean hemagglutination titer of allantoic fluids* of embryos inoculated with virus and injected with			Difference in titers of NaCl and Fr.B groups	
In presence of	No. of passages	Dilution employed	NaCl‡	Fr.B‡		fold	log
mg./embryo				mg./embryo		fold	log
—		Parent strain	10⁻¹	420	1.00	384	0	−0.04
Fr.B	1.00	1	10⁻⁵	128	0.05	65	−2×	−0.30
"	0.05	2	"	152	1.00	128	0	−0.07
NaCl		1	"	129	0.05	4	−33×	−1.52
—		Parent strain	"	512	0.05	"	−128×	−2.11

* Allantoic fluids were harvested 6 days after inoculation.
‡ Injection of NaCl or Fr.B was given 1 hour after virus.

of the parent strain, it should be possible by means of the inhibiting polysaccharide to demonstrate its presence in a single cycle of multiplication.

A large quantity of mumps virus, *i.e.* 10^6 E.I.D., was inoculated into the allantoic sac of each of two groups of embryos. After an interval of 1 hour one group was injected intra-allantoically with 1.0 mg. of Fr.B per embryo, whereas the other group was given 0.1 cc. saline. After incubation at 35°C. for 6 days, the allantoic fluids were removed separately and their hemagglutination titers determined. Two passages were carried out in a manner identical to that described above.

The results obtained are presented in Table II. It will be seen that the virus which was present after a single passage in the presence of Fr.B was capable of multiplication which was not inhibited by the polysaccharide. The variant strain obtained under these conditions in a single passage was found to be relatively stable, and retained its capacity to multiply in unrestricted fashion in the presence of Fr.B even after serial passage in the absence of the polysaccharide. It should be pointed out that a resistant variant

TABLE III

Effect of Capsular Polysaccharide of Friedländer Bacillus, Type B, on Multiplication of Large Inocula of Mumps Virus

1st injection Intra-allantoic	Interval	2nd injection Intra-allantoic	Interval at 35°C.	Mean hem-agglutination titer of allantoic fluids	Difference in titers of NaCl and Fr.B groups	
E.I.D.	*hrs.*		*days*		*fold*	*log*
MV 10^6	3	Saline	3	162		
" "	"	Fr.B 1.0 mg./embryo	"	16	−10×	−1.01
" "	"	Saline	6	213		
" "	"	Fr.B 1.0 mg./embryo	"	192	0	−0.05

* E.I.D. = embryo infectious doses.

was obtained also from the Enders strain[2] of mumps virus in a single passage by means of an identical experimental procedure. Thus it appears that when an inoculum containing a large quantity of mumps virus is employed, there is present in it a sufficient number of variant particles so that they become clearly demonstrable during a single cycle of multiplication. Additional evidence in support of this view is shown in Table III. It will be observed that during the first 3 days after inoculation the virus multiplied less readily in the presence of polysaccharide, and reached a concentration equivalent to only 10 per cent of that found in control embryos. At 6 days, however, there was no difference in the titers. Because of the limitations of available techniques, it is not possible to estimate with any reliability the probable concentration of variant virus particles in the parent strain. However, without making unwarranted assumptions, it appears that a ratio between variant and typical viral particles of the order of $1:10^4$ or lower would conform with the available data. These re-

[2] Received through the courtesy of Dr. John F. Enders, Children's Hospital, Boston.

sults provide an adequate explanation for the earlier finding (2) that quantitative inhibition of multiplication was demonstrable 6 days after inoculation with 10^4 E.I.D. but not with 10^5 E.I.D. of mumps virus.

Disappearance of Variant on Serial Passage in the Absence of Polysaccharide.— It was of interest to determine whether (a) the resistant variant would retain its capacity to multiply in the presence of Fr.B after serial passage, or (b) upon several passages of the variant in the absence of Fr.B. it would disappear, and a strain of virus reappear which would be inhibited by polysaccharide.

TABLE IV

Reappearance of Sensitive Strain on Serial Passage of Resistant Variant of Mumps Virus in the Absence of Fr.B

Serial passage of mumps virus				Mean hemagglutination titer of allantoic fluids* of embryos inoculated with virus and injected with			Difference in titers of NaCl and Fr.B groups	
Strain	In presence of	No. of passages	Dilution employed	NaCl‡	Fr.B‡		fold	log
					mg./embryo			
Parent	—	—	10^{-5}	74	0.05	1	$-74\times$	-1.87
Resistant variant§	Fr.B	13	"	704	1.00	672	0	-0.02
" "	NaCl	1	"	193	0.05	85	$-2\times$	-0.36
" "	"	"	"	"	1.00	40	$-5\times$	-0.69
" "	"	4	"	384	0.05	144	$-3\times$	-0.42
" "	"	"	"	"	1.00	72	$-5\times$	-0.72
" "	"	5	"	1024	0.05	25	$-41\times$	-1.61
" "	"	"	"	"	1.00	18	$-63\times$	-1.80
Parent	—	—	"	339	0.05	16	$-21\times$	-1.33

* Allantoic fluids were harvested 6 days after inoculation.
‡ Injection of NaCl or Fr.B was given 1 hour after the virus.
§ Resistant variant employed after 13 serial passages in presence of Fr.B.

Approximately 10^2 E.I.D. of resistant variant strains, obtained as described above, was inoculated intra-allantoically in groups of embryos. Each embryo received 0.1 cc. of saline intra-allantoically 1 hour after inoculation. Serial passage was carried out with allantoic fluid obtained after 6 days' incubation. The procedure was identical to that employed in the selection of the variant virus except that polysaccharide was not used in the passage series. The individual allantoic fluid with the highest titer was employed as inoculum at each passage, and was diluted 10^{-5}. The ability of the virus to multiply in the presence of 0.05 and 1.0 mg., respectively, of Fr.B was determined after each passage.

The pertinent data from one such experiment are presented in Table IV. The variant virus strain employed was that derived from the passage series

recorded in Table I. This experiment was carried out after the virus had been through 13 serial passages in the presence of polysaccharide. It is evident that after 4 passages in the absence of Fr.B the strain was still resistant to the inhibitory effect of the polysaccharide; significant inhibition of multiplication was not obtained even when as much as 1.0 mg. of Fr.B per embryo was employed. However, after 5 passages in the absence of the carbohydrate, multiplication of the virus was markedly inhibited when as little as 0.05 mg. of Fr.B was used. Further passages showed that this strain remained sensitive to the inhibitory effects of Fr.B. In this respect it was indistinguishable from the parent strain.

The variant strain which had been selected in a single passage in the presence of Fr.B, as shown in Table II, regained sensitivity to the inhibitory effect of the polysaccharide somewhat more rapidly. After 3 serial passages in the absence of Fr.B the multiplication of this strain was inhibited by 0.05 mg. of Fr.B. It is of interest that in each instance the reappearance of sensitivity to the effect of the polysaccharide was as readily demonstrable with 0.05 mg. as with 1.0 mg. of Fr.B.

Rate of Multiplication of Mumps Virus and Its Resistant Variant.—In an earlier study (2) it was shown that mumps virus did not reach maximum concentration in the allantoic fluid until 6 days after the inoculation of 10^2 E.I.D. It appeared important to determine, as precisely as possible, the rate of multiplication of the virus in the allantoic sac, and to compare this with the rate of multiplication of the resistant variant derived from the parent strain. Experiments were carried out simultaneously with the parent and variant strains. With both strains very large, as well as relatively small, inocula were employed. It was expected that when large inocula were given a large number of cells would be infected almost simultaneously, and that this would result in a sharp increase in viral concentration following the so called latent period. In this manner, it was anticipated that information might be gained as to the approximate duration of the period required for the multiplication of mumps virus and the resistant variant in individual cells of the allantoic sac.

Two large groups of 8 day embryos were inoculated intra-allantoically with approximately 10^7 E.I.D. of virus; one group was given the parent strain, the other received the variant strain. After incubation at 35°C. for 24 hours, the hemagglutination titers of the allantoic fluids from 5 or 6 embryos of each group were determined. Similar titrations were carried out with groups of allantoic fluids removed at frequent intervals thereafter. In other experiments 10^2 E.I.D. of either the parent strain or the variant strain was inoculated intra-allantoically in a large group of embryos. After 1 hour the embryos which had received the resistant variant were given 0.05 mg. of Fr.B while those which had received the parent strain were given 0.1 cc. of saline. After 3 days' incubation at 35°C., the hemagglutination titers of the allantoic fluids from 5 embryos of each group were determined. Thereafter, at 24 hour intervals, similar titrations were carried out with groups of allantoic fluids. Two identical experiments were carried out, and the geometric means of the titers obtained at each time interval were computed.

The results of these experiments are shown in Fig. 1. It will be seen that when 10^7 E.I.D. of either the parent strain or the variant was inoculated, the virus
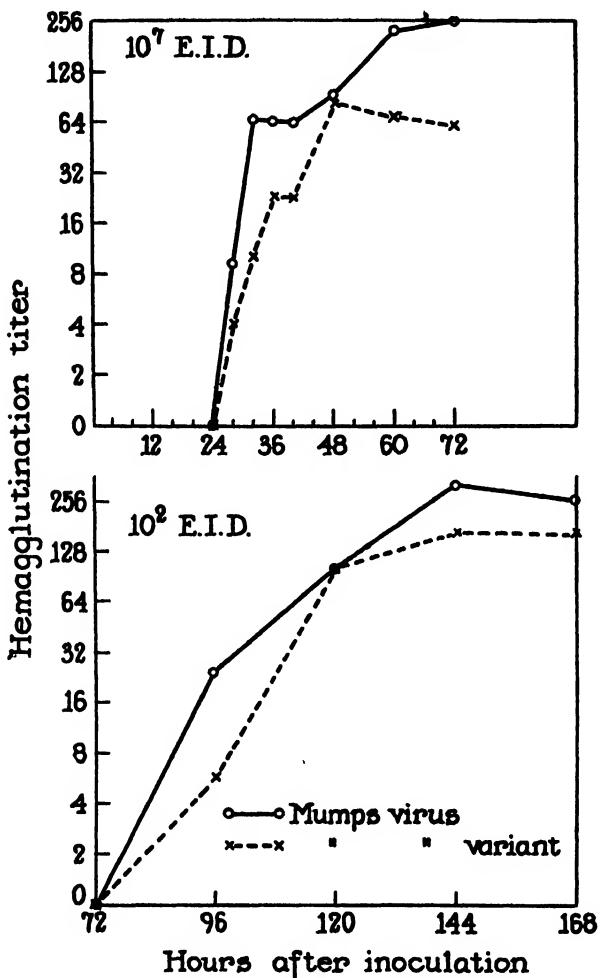
FIG. 1. Rate of multiplication of mumps virus and resistant variant in the allantoic sac. Mean hemagglutination titers of allantoic fluids are plotted against time after inoculation. Upper graph shows results obtained in groups of 5 to 6 embryos after inoculation of 10^7 E.I.D. Lower graph shows combined results of two experiments in groups of similar size after inoculation of 10^2 E.I.D.

was first demonstrable by means of the hemagglutination technique at 28 hours. At 32 hours all allantoic fluids contained detectable concentrations of virus. With the parent strain a concentration plateau appeared at 32 hours and persisted to 40 hours. However, with the resistant variant evidence of such a

plateau did not appear until 36 hours. With both strains evidence of a sharp increase in concentration appeared after 40 hours. It will be noted that when 10^2 E.I.D. was inoculated neither strain was demonstrable at 72 hours and that the parent strain showed a higher concentration than the resistant variant at 96 hours. As was to be expected, no evidence of a plateau was obtained under the experimental conditions employed with the smaller inoculum.

These results indicate that the rates of multiplication of the parent strain and the resistant variant, although not greatly dissimilar, probably are not identical. It appears that the variant strain undergoes multiplication at a slightly slower rate than the parent virus and, as would be expected, this is most evident when large inocula are employed and short time intervals are utilized. It should be pointed out that in a previous study (2) it was shown that hemagglutination is caused by the mumps virus particle itself and that the hemagglutination titer is a measure of viral concentration.

Infectivity of the Resistant Variant.—The infectivity titer of the variant strain was determined in the chick embryo in the presence of polysaccharide. For comparison, a virus titration of the parent strain was carried out simultaneously. Strains were employed which had been subjected to 9 serial passages in parallel; the variant in the presence of Fr.B, the parent in the absence of polysaccharide.

The infected allantoic fluids which were selected for titration experiments had similar hemagglutination titers. Infectivity titers were determined in 8 day embryos in the manner described above. One hour after inoculation of dilutions of the resistant variant 0.05 mg. of Fr.B was injected. The inhibitory effect of Fr.B on each strain was determined at the same time that the titrations were carried out.

The results of this experiment are shown in Table V. The virus infectivity titer of the resistant variant was found to be practically identical to that of the parent strain of mumps virus. It should be emphasized that despite the fact that the variant strain was titrated in the presence of Fr.B it appeared to be equally as infectious as the parent strain.

Immunological Properties of the Resistant Variant.—It was of interest to determine whether the variant strain, which had been obtained by means of a chemical inhibitor, was distinguishable in immunological properties from the parent mumps virus strain. The immunological relationship of the two strains was investigated by means of cross-neutralization and cross-hemagglutinaton-inhibition experiments with specific immune rabbit sera.

Antibody titrations were carried out simultaneously with the resistant variant and the parent strains. To reduce dilution errors, each strain was tested against aliquots from a single series of dilutions prepared from the desired immune serum. In hemagglutination-inhibition titrations final concentrations of 4 hemagglutinating units of virus and 0.5 per cent chick RBC were employed. Readings were made after 1 hour at room temperature; the end point was taken as the highest dilution which gave complete inhibition. In virus neutralization

TABLE V

Infectivity Titers of Resistant Variant of Mumps Virus and Parent Strain in the Chick Embryo

Virus	1st injection Intra-allantoic	2nd injection* Intra-allantoic	Mean hemagglutination titer of allantoic fluids‡	Difference from controls		Infectivity score	Infectivity titer
				fold	log	positive/total	E.I.D.50
Parent strain§	10⁻⁵	NaCl	28			4/4	
" "	"	Fr.B 0.05 mg./embryo	2	−14×	−1.16	1/3	
" "	10⁻⁶	NaCl				4/4	
" "	10⁻⁷	"				2/4	10⁻⁷·⁰
" "	10⁻⁸	"				0/4	
" "	10⁻⁹	"				0/4	
Resistant variant‖	10⁻⁵	NaCl	256			3/3	
" "	"	Fr.B 0.05 mg./embryo	120	−2×	−0.33	4/4	
" "	10⁻⁶	" "				4/4	
" "	10⁻⁷	" "				3/4	10⁻⁷·³
" "	10⁻⁸	" "				0/4	
" "	10⁻⁹	" "				0/4	

* 2nd injection given 1 hour after 1st injection.
‡ Allantoic fluids harvested 6 days after inoculation.
§ After 9 serial passages in presence of NaCl.
‖ After 9 serial passages in presence of Fr.B, 0.05 mg./embryo.

TABLE VI

Neutralization and Hemagglutination-Inhibition Titers of Sera from Rabbits Immunized with Parent Strain or Resistant Variant of Mumps Virus

Immune serum against	Hemagglutination-inhibition titer* vs.		Neutralization titer‡ vs.	
	Parent strain	Resistant variant	Parent strain	Resistant variant
Parent strain...................	1024	1024	600	300
Resistant variant...............	256	256	50	62

* Final concentration of 4 hemagglutinating units of virus employed.
‡ Approximately 10³ embryo infectious doses of virus employed.

experiments a constant quantity of virus, *i.e.* 10³ E.I.D., was used. Serum dilution-virus mixtures were incubated at 37°C. for 30 minutes. Each mixture was inoculated intra-allantoically in a group of 4 embryos. After incubation for 6 days at 35°C. the hemagglutination titers of the allantoic fluids were determined and the virus neutralization titer of the serum was calculated by the method of Reed and Muench (8).

The results of typical antibody titrations with immune rabbit sera against the parent strain and the resistant variant of mumps virus are shown in Table VI. It will be observed that similar antibody titers were obtained with each immune serum irrespective of the viral strain employed. Similar experiments were carried out with sera from other rabbits immunized either with the parent or the variant strain, and in every instance almost identical results were obtained. It appears evident that the resistant variant possesses immunological properties which are not dissimilar from those of the parent strain and it seems probable that the variant is antigenically indistinguishable from the parent strain.

Rate of Reaction of the Resistant Variant with Chicken Erythrocytes.—Björkman and Horsfall (9) demonstrated that stable variants could be obtained from influenza viruses by treatment with lanthanum acetate or by irradiation with ultraviolet light. Such variants differed from the parent virus strains in that their rates of elution from chicken RBC were slower than those of the viruses from which they were derived. Experiments were carried out to determine whether the resistant variant of mumps virus differed from the parent strain in a similar manner. It was found that there was no demonstrable difference between the parent strain and the variant as to the rate or degree of adsorption by chicken RBC as well as in the rate or extent of elution from such erythrocytes.

Concurrent Multiplication of the Resistant Variant and Influenza Virus in the Chick Embryo.—The results of an earlier study (3) indicated that viruses which are affected by the inhibitory action of Friedländer polysaccharides do not show the interference phenomenon with viruses which multiply in an unrestricted manner in the presence of such carbohydrates. As an example, it was demonstrated that multiplication of influenza A or B virus occurred in the allantoic sac when either virus was injected as long as 4 days after infection with mumps virus. Because the multiplication of the resistant variant of mumps virus was as unaffected by the presence of polysaccharide as is the multiplication of influenza viruses (2), the possibility that they might show interference was investigated.

Experiments were carried out in a manner identical to that employed in previous studies (3). The resistant variant was inoculated into the allantoic sac in groups of embryos and allowed to multiply for 4 days. Either the PR8 or Lee strain of influenza virus was then inoculated and incubation was continued for 2 additional days. The concentration of each of the viruses present in the allantoic fluids was determined by the hemagglutination technique in the presence of appropriate specific immune sera as described previously (3).

It was found that preexisting infection of the chick embryo with the variant of mumps virus did not prevent infection with either influenza A or B virus. In this respect, the resistant variant is similar to the parent strain; both are capable of concurrent multiplication with influenza viruses in the allantoic sac.

It has not been possible to devise a procedure which would be satisfactory to test the possibility that the resistant variant and the parent strain of mumps virus cause reciprocal interference.

DISCUSSION

That a variant can be obtained from mumps virus appears clear from the results of this study. Demonstration of the presence of the variant depended upon the availability of a chemical inhibitor of mumps virus multiplication. The property which clearly distinguishes the variant from the parent strain is its capacity to multiply as readily in the presence as in the absence of Friedländer capsular polysaccharides. It seems probable that this property is highly significant, and reflects an important difference in the biochemical mechanism by which the variant undergoes multiplication in the host cell as compared to that required by the parent strain.

Because the variant is consistently demonstrable after a single cycle of multiplication in the presence of polysaccharide, it appears that it is commonly present; that it is a naturally occurring variant which arises spontaneously, and does not develop as a result of a stimulus provided by the presence of polysaccharide. If this is the case, then it follows that mumps virus is not represented by individual viral particles, all of which are identical. A small proportion of the viral population, possibly of the order of 0.01 per cent, appears to possess at least one property which sharply distinguishes it from the bulk of the population. This property, the capacity to multiply readily in the presence of Friedländer polysaccharide, makes possible both the demonstration of the presence of the variant and its separation from the typical viral population.

The probable existence of inhomogeneity among animal virus populations was called to attention previously (9) in a study which showed that variants could be separated from influenza viruses by means of chemical or physical agents. In the case of influenza viruses the property which distinguished the variants, slow rate of elution from RBC, could not have been predicted on the basis of the agents, lanthanum acetate or ultraviolet light, employed to separate them. On the other hand, in the case of the resistant variant of mumps virus disclosed in this study, the distinguishing property of the variant is predictable, although undirected, and appears to be related in a specific manner to the chemical inhibitor, Friedländer polysaccharide, used to separate it.

There is as yet no means by which an entirely "pure line" strain of an animal virus can be obtained with certainty. With available techniques it is not possible to develop a viral population known to have originated from a single particle of an animal virus. As a consequence, variant strains derived from animal viruses probably cannot be considered to be composed solely of variant particles. In all likelihood, they represent mixtures, are inhomogeneous, and contain both variant and parent particles in different proportions. Serial passage

of such a mixture in the absence of the substance which favors the appearance of the variant would be expected to result in the reappearance of the parent strain if the parent possesses a slight advantage over the variant. The finding that the parent strain of mumps virus has a slightly more rapid rate of multiplication than the resistant variant indicates that it possesses such an advantage, and provides a probable explanation for the reappearance of the parent strain on serial passage of the variant in the absence of polysaccharide. However, if it is assumed that prolonged serial passage in the presence of inhibitory polysaccharide results in complete elimination of the parent virus, *i.e.* in the development of a "pure line" variant strain, it is necessary to consider an alternative hypothesis. Under these circumstances, so called reverse variation, with reappearance of the parent strain, would provide an explanation for the findings. Because of the rapidity with which the parent strain reappeared, it seems improbable that reverse variation occurred.

Variation with viruses, plant, bacterial, or animal, is a well known phenomenon which has been studied extensively. A large body of experimental data indicates that viruses, like other infectious agents, may show variation with respect to almost any property. Some of the most clearly established examples of variation are concerned with differences in pathogenicity, host range, and antigenic composition. It may be pointed out that these are the properties of viruses which, because of the limitations of available techniques, have been subjected to most intensive study. It should be emphasized that variation relative to these properties is unforeseeable and, although readily demonstrable, not subject to precise experimental control.

The resistant variant of mumps virus revealed in this study appears to represent a new type of viral variant, obtainable at will, with properties which can be anticipated in terms of the chemical inhibitor of multiplication used for selection of the variant. So far as we can discover, the only other example of this type of variation is that recorded by Foster (10) who found that "mutants" selected from T₄ and T₆ bacteriophage with proflavine showed somewhat higher tolerance to the inhibitory effects of the chemical than the parent viruses. It is obvious that the resistant variant of mumps virus is, in a formal sense, analogous to bacterial mutants which are resistant to antimicrobial agents. There is, however, an important difference which should be stressed. The available evidence (2, 11) indicates that Friedländer polysaccharide has no direct effect upon mumps virus itself, and suggests that the inhibition of multiplication obtained with the carbohydrate is the result of an effect upon the host cell.

That a variant of mumps virus is capable of unrestricted multiplication in the presence of inhibitory quantities of polysaccharide has implications bearing on the mechanism of inhibition. In terms of present views on the manner of viral multiplication there appear to be four steps in the process which in theory could be affected: (1) Polysaccharide might prevent virus-cell combination;

i.e., block or compete with "receptors" at the cell membrane. Previous studies with both PVM (12) and mumps virus (2, 11) indicate that this hypothesis is untenable. However, Green and Woolley (13) suggested that apple pectin inhibited influenza A virus by such a mechanism. In the case ι the esistant variant of mumps virus it is evident that virus-cell combination occu.. whether or not polysaccharide is present and there appears to be no reason to raise the unlikely possibility that some different cell "receptor" is utilized by the variant. (2) Polysaccharide might prevent invasion or penetration of the cell by virus. Earlier studies make this theory improbable; with mumps virus (2), as too with PVM (12), inhibition is obtained after the viral concentration has reached a level high enough to indicate that almost all susceptible cells are already infected. Other workers (14, 15) have considered that the results they obtained on inhibition of bacteriophage and influenza A virus with pectins could be explained in this manner. With the resistant variant of mumps virus it is apparent that invasion of the cell is accomplished despite the presence of polysaccharide, and it seems unnecessary to assume that the variant penetrates the cell in a manner different than the parent virus. (3) Polysaccharide might prevent release of virus from the infected cell after multiplication occurs. This hypothesis could explain the reduced concentration of mumps virus in allantoic fluid but would not explain the fact that finely ground tissues show, with either PVM (12) or mumps virus (16), a similarly reduced viral concentration when polysaccharide is employed. Moreover, the resistant variant is released from infected cells into the allantoic fluid as completely when polysaccharide is present as when it is absent. (4) Polysaccharide may, as was suggested previously (2, 3, 12), block or compete with an intracellular process which is required by the virus during multiplication. This hypothesis, in contrast to those discussed above, appears to conform with available information and is supported by the results obtained in the present study. To bring the resistant variant into line with this theory it is only necessary to assume that it has certain biochemical requirements different from the parent virus. Such an assumption is not at odds with well established facts bearing on the mechanism of variation with other infectious agents. If inhibition of mumps virus multplication is in fact due to blockage of an essential cellular metabolic step by polysaccharide, then it would be predicted that the resistant variant either makes use of some metabolic step other than that affected by the polysaccharides of Friedländer bacilli or does not require that step.

SUMMARY

Serial passage of mumps virus in the presence of inhibitory quantities of the capsular polysaccharide of Friedländer bacillus type B results in the appearance of a variant strain of the virus. Multiplication of the variant virus is not inhibited by the polysaccharide. A similar resistant variant is obtained with

polysaccharide in a single cycle of multiplication when very large inocula of mumps virus are employed. The resistant variant is indistinguishable from the parent strain as to infectivity, reactivity with erythrocytes, and immunological properties, but appears to have a somewhat slower rate of multiplication. Serial passage of the resistant variant in the absence of polysaccharide results in the reappearance of a sensitive strain. It is suggested that mumps virus populations are inhomogeneous; that naturally occurring variants are present in such populations and possess distinctive properties; that the use of a chemical inhibitor of mumps virus multiplication makes possible the selection of a variant possessing a predictable property. The findings are discussed in relation to the mechanism of inhibition of mumps virus multiplication by polysaccharide.

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A LABILE COMPONENT OF NORMAL SERUM WHICH COMBINES WITH VARIOUS VIRUSES. NEUTRALIZATION OF INFECTIVITY AND INHIBITION OF HEMAGGLUTINATION BY THE COMPONENT

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During an investigation of the etiology of an epidemic of a mild infectious disease, for convenience termed "epidemic myalgia," it was observed that a heat labile component in serum obtained during convalescence neutralized Newcastle disease virus. It was noted that serum stored at 4°C. for more than 2 weeks was no longer able to protect chick embryos from infection with this virus, whereas serum held in the frozen state at −28°C. retained such activity. The importance of this phenomenon seemed considerable on both a practical and theoretical level.

A number of workers have observed that certain thermolabile components of serum' either alone or in conjunction with immune antibodies, may neutralize the infectivity of various viruses. Gordon (1) observed that the addition of fresh guinea pig serum to heated serum of a rabbit immunized with vaccinia virus increased the neutralization titer against vaccinia virus. Douglas and Smith (2) also noted a heat labile component in normal rabbit blood, plasma, and serum which inhibited infection of rabbits with vaccinia virus, and which was not active when sodium citrate was employed as an anticoagulant. That the activity of immune serum against Rous sarcoma virus could be enhanced by the addition of fresh guinea pig serum was pointed out by Mueller (3) who interpreted this to be an activity of complement. More recently Morgan (4) and Whitman (5) both demonstrated that unheated immune rabbit serum neutralized Western equine encephalomyelitis virus in higher dilutions than did the same serum heated at 56°C. for 30 minutes. The addition of fresh, unheated guinea pig serum (4, 5), monkey, or human serum (5) to inactivated immune serum increased neutralization. Morgan (4) believed the increased activity resulting from the addition of normal guinea pig serum to be the result of the enhancement of antibody activity by complement. Leymaster and Ward (6) have demonstrated higher mumps virus neutralization titers when immune sera were not heated. The addition of normal, unheated human or monkey serum also enhanced the neutralization titers of heat-inactivated human sera (7). It has been pointed out recently by Smith and Westwood (8) that unheated sera of guinea pigs neutralized influenza A virus.

It is the purpose of this paper to show that there is present in the serum of normal human beings, guinea pigs, rabbits, or mice a heat labile component which neutralizes the infectivity of a number of viruses and inhibits hemagglu-

tination of chicken erythrocytes by these agents; that the serum component produces these effects by combining with the viruses studied; that calcium ions are necessary for serum component–virus combination to take place; that the labile component is probably a protein or protein complex; that the thermolabile component is not identical with hemolytic complement.

Materials and Methods

Viruses.—The following viruses were employed: influenza A, PR8 strain; influenza B, Lee strain; Newcastle disease; and mumps which will be referred to as PR8, Lee, NDV, and MV, respectively. Each virus was propagated in the allantoic sac of chick embryos 7 to 12 days of age. The methods of cultivation and storage were identical to those previously described (9).

Virus Infectivity and Hemagglutination Titrations.—The methods employed were identical to those used previously (9). Infectivity titrations were carried out with serial tenfold dilutions of infected allantoic fluid inoculated into the allantoic sac of chick embryos of appropriate age. Hemagglutination titrations were performed with serial twofold dilutions in 0.85 per cent NaCl buffered at pH 7.2 (0.01 M phosphate), hereafter referred to as saline, and an equal volume of a 1 per cent suspension of chicken RBC. The results of all titrations are expressed in terms of final concentrations.

Virus Neutralization Titrations.—Twofold dilutions of serum were made in saline, and to each was added a constant quantity of virus (usually 10^2 to 10^3 embryo infectious doses, E.I.D.). After a period of incubation, 0.1 or 0.2 cc. of each mixture was injected intra-allantoically into each of 4 embryos of appropriate age. The hemagglutination titer of each allantoic fluid was determined after incubation at 35°C. for a period dependent upon the virus employed.

Hemagglutination-Inhibition Titrations.—Twofold dilutions of serum were prepared in saline, and to each was added a constant quantity of virus; 16 hemagglutinating units usually was employed. After incubation for 15 or 30 minutes in a water bath at 37°C., an equal volume of a 1 per cent suspension of chicken RBC was added. The titer was taken as the highest dilution in which there was complete inhibition of hemagglutination.

Sera.—Human, guinea pig, rabbit, and mouse sera were employed. Guinea pig and mouse sera were obtained from groups of at least 4 animals and pooled. Blood was obtained from children 3 to 4 years of age with no history of mumps or influenza. All blood specimens were allowed to clot at 4°C. The serum was separated promptly, then stored in 1 to 2 cc. volumes in nitrose cellulose tubes at −28°C.

Complement Titrations.—Titrations were carried out with various guinea pig serum fractions as well as fresh guinea pig serum by employing decreasing quantities of a 1:20 or 1:30 dilution. Two units of anti-sheep hemolysin and a 3 per cent suspension of sheep RBC were employed. All suspensions and dilutions were made in 0.85 per cent NaCl containing 0.1 gm. $MgSO_4$ per liter. Complement titers are expressed as the smallest quantity of serum in which complete hemolysis of the sensitized sheep RBC was brought about.

EXPERIMENTAL

Prevention of Virus Infection of the Chick Embryo by a Heat Labile Component of Serum.—Sera obtained from patients ill with a mild infectious disease were examined for antibodies against various viruses. Preliminary tests showed that unheated sera obtained during the acute phase of the illness did not prevent infection of chick embryos with Newcastle disease virus (NDV), whereas unheated sera obtained during convalescence did prevent such infection. As

it happens, the acute phase sera had been stored at 4°C. for 10 to 18 days before being frozen and stored at −28°C., whereas the convalescent sera were stored at the latter temperature immediately after separation. This question arose: was neutralization of NDV a result of the development of specific antibodies or was it due to the presence of an unstable component of serum other than antibody which was capable of inactivating the virus?

TABLE I

Neutralization of Newcastle Disease Virus by a Heat Labile Component of Human Serum

Mixture* 0.2 cc. intra-allantoic				Hemagglutination titer allantoic fluids‡				Serum neutralization titer
Human serum			NDV virus	A	B	C	D	
Storage 4°C.	Date obtained	Dilution						
days	*1948*		*E.I.D.§*					
0¶	Aug. 8	1:2	10³	0	0	0	0	
		1:4	"	0	0	0	16	1:5
		1:8	"	32	2	64	4	
		1:16	"	64	32	128	8	
16	" "	1:2	"	128	64	>128	32	0
		1:4	"	>128	128	128	64	
8	" 16	1:2	"	4	0	16	16	0
		1:4	"	16	16	64	16	
0	Sept. 10	1:2	"	0	0	0	0	
		1:4	"	0	4	0	0	1:5
		1:8	"	64	32	32	16	
		1:16	"	128	128	128	64	

* Held at room temperature for 30 minutes before inoculation.
‡ Expressed as the reciprocal.
§ E.I.D. = embryo infectious doses.
¶ Heparinized plasma stored at −28°C.

Human sera and heparinized plasma stored at −28°C., as well as sera stored for varying periods at 4°C. before being frozen, were employed in virus neutralization experiments, performed as described above, with NDV, PR8, Lee, and MV. In each experiment unheated serum and serum heated at 56°C. for 30 minutes were used.

The results of a typical experiment with human serum or heparinized plasma and NDV are presented in detail in Table I. Serum stored at 4°C. for 16 days did not prevent infection with 10³ E.I.D. of virus, whereas plasma obtained simultaneously and stored at −28°C. completely neutralized the agent. Serum obtained later and stored at −28°C. also neutralized the virus. Serum stored at 4°C. for only 8 days did not prevent infection but the hemagglutination

titers of the infected allantoic fluids were reduced. Unheated sera from 5 other patients with a similar disease and from 3 healthy persons were studied with NDV, and corresponding results were obtained; the neutralization titers varied from 1:5 to 1:21 when 10^2 E.I.D. of virus was employed. In each instance serum heated at 56°C. for 30 minutes did not neutralize the virus.

When dilutions of NDV were mixed with unheated human serum, it was found that relatively large amounts of virus were neutralized. The results of one such experiment are presented in Table II, and show that as much as 10^4 E.I.D. of NDV was neutralized by the unheated serum; i.e., the unheated serum had a neutralization index of 10,000 relative to the heated specimen.

Because adult human sera commonly contain specific antibodies against the viruses of influenza A, B, and mumps, they were unsatisfactory for this study. However, sera were obtained[1] from 2 children, 3 and 4 years old, respectively, who had no history of infection with these agents, and studies were carried out

TABLE II

Infectivity Titer of Newcastle Disease Virus in Presence of Unheated and Heat-Inactivated Human Serum

Mixture*		Incubation at 37°C.	Virus infectivity titer	Neutralization index
NDV	Human serum			
0.3 cc.	*0.3 cc.*	*min.*		
Serial dilutions	56°C., 30 min.	30	$10^{-7.8}$	
" "	Unheated	"	$10^{-3.7}$	10,000

* 0.1 cc. intra-allantoic per embryo.

in a manner identical to that described above. The results of experiments with these sera and mumps virus are summarized in Table III. The mean neutralization titer of the children's unheated sera was 1:54 when 10^2 E.I.D. of mumps virus was used, whereas the heated sera did not neutralize the virus at a dilution of 1:2. Unfortunately, the children's sera contained heat stable components, probably specific antibodies, which neutralized both influenza A and B viruses, and therefore it was not possible to test for a thermolabile component with these agents.

Inactivation of Various Viruses by Serum of Normal Animals.—In order to learn more about the heat labile component of normal human serum which inactivates NDV and mumps, it seemed advisable to determine: (1) whether a similar component was present in the serum of various laboratory animals, and (2) whether such a component, if present, would inactivate viruses other than NDV and mumps. Serum was obtained from normal guinea pigs, rabbits.

and mice, and employed in experiments similar to those described above. The viruses of Newcastle disease, mumps, influenza A, and influenza B were used. The results of these experiments are summarized in Table III, and expressed as the mean of the neutralization titers obtained in at least two experiments. It is evident that a heat labile neutralizing component is present in both guinea pig and rabbit sera. The unheated guinea pig sera neutralized Lee, PR8, and MV, as well as NDV; rabbit sera inactivated NDV in comparable dilutions, but Lee and PR8 were neutralized only in low dilutions of serum; mouse sera did not contain the heat labile component in sufficient quantity to neutralize either NDV or Lee. It should be emphasized that in every instance when normal animal sera were heated at 56°C. for 30 minutes, the capacity to neutralize each of the viruses disappeared.

TABLE III

Neutralization of Newcastle Disease, Influenza A, B, and Mumps Viruses by a Heat Labile Component of Human, Guinea Pig, and Rabbit Sera

Serum	Mean neutralization titers of unheated sera against indicated virus*			
	NDV‡	PR8‡	Lee‡	MV§
Human..........................	1:10	Ab.¶	Ab.¶	1:54
Guinea pig.....................	1:19	1:21	1:13	1:48
Rabbit.........................	1:13	1:2	1:4	<1:8
Mouse.........................	<1:2	—	<1:2	—

* In every instance in which a titer is given the heated serum failed to cause any neutralization.
‡ 10⁸ E.I.D. employed.
§ 10³ " "
¶ Specific antibodies present.

It was found that neutralization of virus infectivity was demonstrable with unheated normal serum when mixtures were injected without preliminary incubation, and the titers obtained were as high as when mixtures were incubated at room temperature for 30 minutes. A twofold increase in titer occurred when serum-virus mixtures were incubated for 30 minutes in a water bath at 37°C. There was no further increase when the period of incubation was increased to 120 minutes at room temperature.

Inhibition of Viral Hemagglutination by a Heat Labile Component of Serum.— In order to investigate more thoroughly neutralization of viruses by a heat labile component of normal serum, it was of importance to attempt to extend the studies by *in vitro* methods. The capacity of unheated serum to inhibit hemagglutination of the viruses used in neutralization experiments was determined.

The experiments were carried out with two different methods: (1) the hemagglutination-inhibition technique with dilutions of serum, as described above, and (2) undiluted serum and virus were mixed, incubated for 15 or 30 minutes at 37°C., and then the hemagglutination titer of the mixture was determined. Most experiments *in vitro* were carried out with Lee virus, but PR8, MV, and NDV were also employed.

For purposes of clarity the results of experiments with each of the *in vitro* inhibition methods are presented in Tables IV and V. Table IV shows the

TABLE IV

Inhibition of Hemagglutination with Lee Virus by a Heat Labile Component in Guinea Pig Serum

Mixture*		Hemagglutination with serum dilutions							Hemagglutination-inhibition titer
Guinea pig serum dilutions	Lee virus	4	8	16	32	64	128	256	
Unheated...................	4 units‡	0	0	0	0	±	2	3	1:64
56°C., 30 min..............	" "	2	2	3	3	3	3	3	0

* Incubated at 37°C. for 30 minutes before addition of 1 per cent RBC.
‡ Final concentration.

TABLE V

Combination of Lee Virus and a Thermolabile Component in Guinea Pig Serum

Mixture*		Hemagglutination with dilutions of mixture										Hemagglutination titer of mixture
Guinea pig serum undiluted	Lee virus	4	8	16	32	64	128	256	512	1024	2048	
Unheated..............	1024 units	0	0	0	0	0	0	0	0	0	0	0
56°C., 30 min..........	" "	4	4	4	3	3	3	3	3	2	0	1:1024

* Incubated for 30 minutes at 37°C. before dilution and addition of 1 per cent RBC.

inhibition of hemagglutination of chicken RBC by a small amount of Lee virus in the presence of dilutions of unheated guinea pig serum. The same serum heated at 56°C. for 30 minutes showed no inhibition. Table V points out the extent of inhibition obtained with undiluted guinea pig serum and a large amount of Lee virus. The unheated serum completely inhibited hemagglutination on dilution of the mixture, whereas the heated serum did not.

Table VI shows a summary of the results obtained in similar *in vitro* inhibition experiments with normal human, guinea pig, and mouse sera and four viruses: NDV, Lee, PR8, and MV. Because of the presence of heat stable inhibitors, rabbit sera were unsatisfactory in these experiments and human sera could only be employed with NDV. It will be noted that both guinea pig and

mouse sera contain a heat labile component which inhibits hemagglutination of each of the viruses tested, and that unheated human serum inhibits hemagglutination of NDV, whereas heated serum does not. Certain guinea pig and mouse sera in low dilutions also contain a stable inhibitor of viral hemagglutination which is not inactivated by heating at 56°C. The stable inhibitor is most readily demonstrated when Lee or PR8 which has been heated at 56°C. for 30 minutes is employed (10).

It was found that inhibition was demonstrable immediately after mixing virus and unheated serum; that the extent of inhibition increased to a maximum value after incubation of the mixture for 15 minutes at 37°C.; that incubation for as long as 3 hours at 37°C. neither increased the degree of inhibition

TABLE VI

Inhibition of Hemagglutination with Newcastle Disease, Influenza A, B, and Mumps Viruses by a Heat Labile Component of Human, Guinea Pig, and Mouse Sera

Serum	Hemagglutination-inhibition titer *vs.*			
	NDV*	PR8*	Lee*	MV*
Human—unheated	1:16	—	—	—
" —heated‡	<1:4	—	—	—
Guinea pig—unheated	1:64	1:128	1:64	1:128
" " —heated‡	<1:8	1:8	<1:4	1:8
Mouse—unheated	1:64	1:64	1:256	1:64
" —heated‡	<1:8	<1:8	<1:8	<1:8

* Final concentration of 4 hemagglutinating units employed.
‡ 56°C. for 30 minutes.

nor brought about separation of the heat labile serum component and virus. It is of interest that not only does virus combine with serum component so that hemagglutination is not brought about by virus, but also virus inactivated by this component does not alter "virus receptors" of erythrocytes; fresh virus is adsorbed by cells treated for long periods with the virus-serum component combination.

Quantitative Factors Concerned with Neutralization and Hemagglutination-Inhibition Titrations.—The relationship between the quantities of virus and serum employed in *in vivo* and *in vitro* titrations of the heat labile serum component was studied.

The experiments were carried out with the Lee virus and a fresh pool of normal guinea pig serum. Hemagglutination-inhibition titrations were carried out with varying quantities of virus. In order to reduce dilution errors to a minimum, a single series of serum dilutions was prepared and aliquots of each dilution were tested against different quantities of virus to give

final concentrations of 1, 2, 4, and 8 hemagglutinating units. Following incubation of the mixtures at 37°C. for 30 minutes, a 1 per cent suspension of chicken RBC was added.

Virus neutralization titrations were carried out as described above. A single series of serum dilutions was used, and an equal volume of Lee virus varying in quantity from 10^2 to 10^5 E.I.D. was added to an aliquot of each dilution.
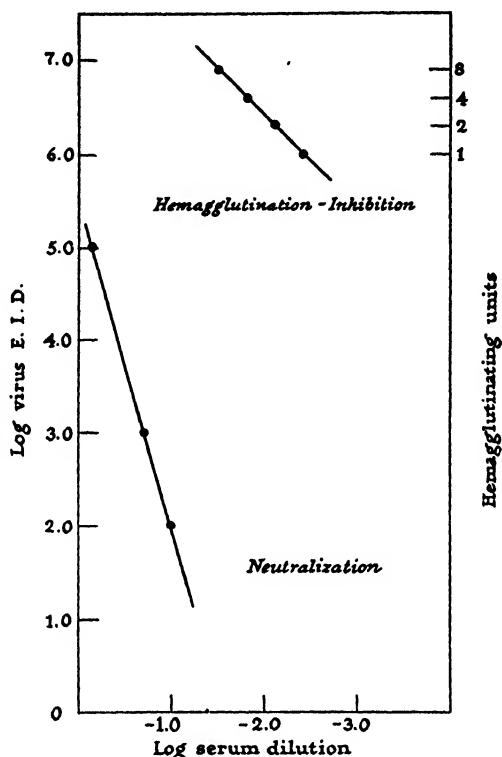
FIG. 1. Linear relationship between the quantity of Lee virus and the neutralization titer as well as the hemagglutination-inhibition titer of unheated guinea pig serum. In neutralization titrations 50 per cent end points were determined with at least 5 serum dilutions. Each serum-virus mixture was inoculated in a group of 4 chick embryos.

The results of these experiments are presented graphically in Fig. 1. There is a linear relationship between the quantity of virus used and the dilution of unheated serum required to inactivate it both *in vivo* and *in vitro*. However, it is important to note that for hemagglutination-inhibition the slope of the line is 1, *i.e.* a twofold decrease in the quantity of virus employed causes a twofold increase in the serum dilution end point, whereas for virus neutralization the slope of the line is much steeper, *i.e.* 3.8, indicating that comparatively large variations in the quantity of virus are required to alter significantly the neutralization titer. These quantitative virus-serum component relationships are

not remarkably different from those which have been found in similar experiments with virus and specific antibody (11, 12).

Inactivation of Serum Component.—One of the distinguishing properties of the normal serum component under study is its lability to heat and upon storage at 4°C. The rate of inactivation of the component during storage at 4°C. and on heating at 56°C. was investigated.

The results of an experiment on heat inactivation of the component in guinea pig serum are presented in Fig. 2. The time of heating in a 56°C. water bath is plotted against the hemagglutination-inhibition titer determined with Lee virus. About 75 per cent of the component was inactivated in 10 minutes, and complete inactivation had occurred after 30 minutes at this temperature.
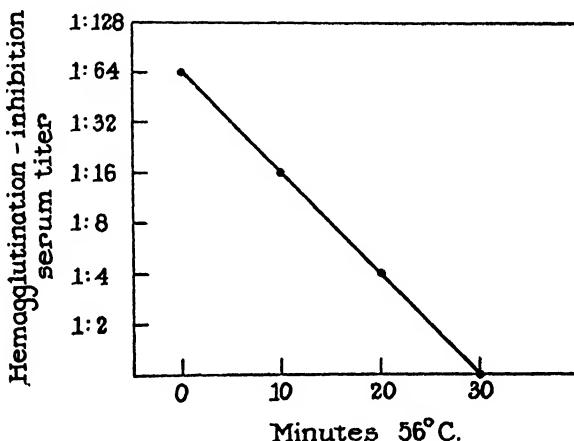
FIG. 2. Relation between the time of heating at 56°C. and the degree of inactivation of guinea pig serum component. Hemagglutination-inhibition titrations were carried out with 4 units of Lee virus.

When guinea pig serum was stored at 4°C., the capacity to neutralize Lee virus in the chick embryo was completely inactivated in 18 hours, but the capacity to inhibit hemagglutination by this virus did not begin to diminish until 7 days of storage, and the inhibitory property was still present, although in very low titer, after 3 weeks. As was pointed out above, the component in human serum which prevents the infection of chick embryos with NDV is inactivated slowly at 4°C. When human serum was tested at regular intervals after storage at 4°C., it was found that inactivation of the neutralizing component commenced after 7 days and was complete after 15 days.

Mechanism of Inactivation of Virus by Serum Component.—The data presented above suggested that virus inactivation by the heat labile serum component was a result of either: (1) stable combination between serum component and virus, or (2) destruction of virus by the serum component, perhaps

enzymatically. That the component acted directly on the virus and not on the host cells was indicated by the finding that a mixture of virus and serum showed no hemagglutination when diluted, and that there was a quantitative relationship between virus and serum component in both neutralization and hemagglutination-inhibition experiments. Attempts were made to obtain further data concerning the mechanism of viral inactivation by the component.

Undiluted fresh guinea pig serum was mixed with an equal volume of NDV diluted 10^{-1} in sterile normal horse serum. A similar mixture of serum which had been heated at 56°C. for 30 minutes and NDV was prepared. Following incubation of the mixtures at 37°C. for 30 minutes each was initially diluted 1:5 following which serial tenfold dilutions were made in broth, and infectivity titrations were carried out in embryos as described above.

TABLE VII

Inactivation of Newcastle Disease Virus by a Thermolabile Component of Guinea Pig Serum

Mixture held at 37°C., 30 min.		Constituents of inoculum final dilutions		Embryo infectivity score	Virus infectivity titration end point E.I.D.₅₀
Guinea pig serum	NDV dilution	Serum	NDV		
				positive/total	
Heated*	10^{-1}	10^{-5}	10^{-6}	4/4	
		10^{-6}	10^{-7}	4/4	
		10^{-7}	10^{-8}	4/4	$10^{-8.5}$
		10^{-8}	10^{-9}	0/4	
Unheated	"	10^{-3}	10^{-4}	4/4	
		10^{-4}	10^{-5}	3/4	$10^{-5.5}$
		10^{-5}	10^{-6}	1/4	
		10^{-6}	10^{-7}	0/4	

* 56°C. for 30 minutes.

The results of this experiment are shown in Table VII. The infectivity titer of NDV which had been mixed with unheated serum was 1,000 times less than that of the virus which had been mixed with heated serum. It will be noted that at a virus dilution of 10^{-7} the serum dilution was 10^{-6}, and yet each embryo was protected from infection with the mixture containing unheated serum. These results indicate that inactivation of NDV by the heat labile component is a result of an effect of the component on the virus itself, and serve to substantiate the results obtained with unheated serum-virus mixtures by the hemagglutination technique.

In order to obtain more direct evidence bearing on the question: is virus inactivation a result of combination with the serum component or a result of enzymatic inactivation by it, the following experiment was carried out.

To 0.8 cc. of fresh guinea pig serum was added 0.8 cc. of Lee-infected allantoic fluid. After incubation at 37°C. for 30 minutes, an aliquot of 0.8 cc. was removed, and to the remainder

of the mixture was added another 0.8 cc. of Lee virus. The mixture was incubated for 30 minutes. This procedure was repeated a third time, and with each aliquot obtained the amount of residual serum component was determined in the following manner: twofold dilutions of each aliquot were made in saline, to each dilution was added an equal volume of Lee virus, and mixtures were incubated at 37°C. for 30 minutes. The hemagglutination titer of each mixture then was determined in the usual manner. Appropriate unheated and heated serum controls were included.

The results of this experiment are presented graphically in Fig. 3 in which the hemagglutination titer of each mixture is plotted against the final concen-
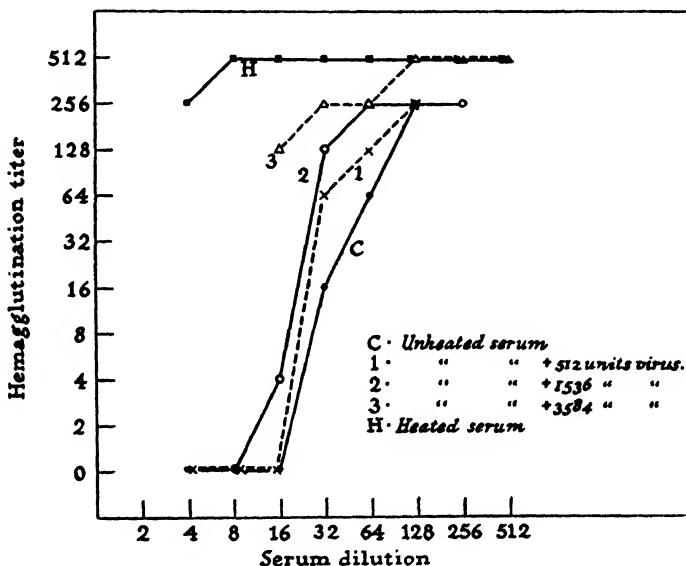
FIG. 3. Effect of adding increasing quantities of Lee virus to unheated guinea pig serum upon the capacity of the serum to cause inhibition of hemagglutination by Lee virus. After the serum had been mixed with the amounts of virus indicated, serial dilutions of the mixtures were prepared, and 512 units of fresh Lee virus was added to each dilution. The hemagglutination titer of each mixture is plotted against the dilution of serum present.

tration of serum present. The control unheated serum and the serum to which only 1 volume of virus had been added completely inhibited 512 hemagglutinating units of Lee virus at a dilution of 1:16, whereas the serum to which 2 volumes of virus had been added inhibited hemagglutination by 128 units of virus at an equivalent dilution. Serum which had been mixed with 3 volumes of virus inhibited only 4 hemagglutinating units of virus at a dilution of 1:16.

These results clearly indicate that Lee virus combines with the heat labile serum component, and prevents the component from combining with viral particles which are added subsequently. When sufficient virus is added to unheated serum, the combining capacity of the component is saturated.

Effect of Various Electrolytes on Serum Component–Virus Combination.— Certain electrolytes have been shown to play an important rôle in various virus-RBC (13–15) and virus-tissue component combinations (15) as well as in many serological reactions (16, 17). The effect of some electrolytes upon heat labile serum component–virus combination was studied.

The electrolyte solutions employed were: 0.85 per cent NaCl buffered at pH 7.2 (0.01 M phosphate), 0.85 per cent NaCl, 0.1 per cent CaCl₂ in 0.85 per cent NaCl, 0.01 per cent MgSO₄ in 0.85 per cent NaCl, 2.5 per cent sodium citrate, and 1.0 per cent sodium oxalate. Hemagglutination-inhibition titrations were carried out using each of these solutions as diluent with guinea pig serum. A final concentration of 4 units of Lee virus was employed.

The results of typical experiments are summarized in Table VIII. Sodium citrate or oxalate completely prevented virus-serum component combination,

TABLE VIII

Effect of Various Electrolytes on Serum Component–Virus Combination

Guinea pig serum dilutions	Diluent	Lee virus	Hemagglutination-inhibition titer*
Unheated	0.85 per cent NaCl; 0.01 M phosphate	4 units	64
"	" " " "	" "	64
"	2.5 " " sodium citrate	" "	<8
"	1.0 " " " oxalate	" "	<8
"	0.1 " " CaCl₂ in 0.85 per cent NaCl	" "	64
"	0.01 " " MgSO₄ " " " " "	" "	64
Heated‡	0.85 " " NaCl; 0.01 M phosphate	" "	<8

* Expressed as the reciprocal.
‡ 56°C. for 30 minutes.

which suggested that calcium or magnesium ions were necessary for serum component–virus combination to take place. It will be noted that there was no difference in the hemagglutination-inhibition titers when 0.85 per cent NaCl was the diluent and phosphate, calcium, or magnesium ions were present.

To determine whether calcium or magnesium ions were necessary for the reaction, guinea pig serum was dialyzed at 4°C. for 16 hours against 1500 volumes of either 0.85 per cent NaCl or 0.1 per cent CaCl₂ in 0.85 per cent NaCl. Following dialysis the hemagglutination-inhibition titers of the serum specimens were determined. The dialyzed serum was diluted in CaCl₂ and MgSO₄ solutions as well as phosphate-buffered saline. The results are summarized in Table IX. Serum dialyzed against saline appeared to lose 75 per cent of its inhibitory activity, but full activity was restored upon the addition of calcium ions although not upon the addition of magnesium ions. Dialysis against a 0.1 per cent CaCl₂ solution caused no loss of inhibitory activity.

Dissociation of Serum Component–Virus Combination.—Combination of the viruses of influenza A and B, mumps, and Newcastle disease with heat-resistant inhibitors present in human, rabbit, or ferret sera (18) as well as normal allantoic fluid (19) or egg white (20) is not stable, and spontaneous dissociation with inactivation of such inhibitors occurs after relatively short periods of incubation (21). However, dissociation of virus and the heat labile serum component does not occur on incubation; 24 hours after preparing a mixture, virus was still not demonstrable by the hemagglutination technique.

Because calcium ions appear necessary for combination between the heat labile serum component and virus, it seemed possible that the addition of sodium citrate to a serum component–virus mixture would remove the available

TABLE IX

Effect of Dialysis and Addition of Calcium on Activity of Thermolabile Serum Component

Serum dialyzed against*	Period dialysis at 4°C.	Dialyzed serum diluted in	Hemagglutination-inhibition titer‡
	hrs.		
None	—	Phosphate buffered 0.85 per cent NaCl	64
0.85 per cent NaCl	16	" " " " " "	16
" " " "	"	0.01 per cent $MgSO_4$ in 0.85 per cent NaCl	16
" " " "	"	0.1 " " $CaCl_2$ " " " " "	128
0.1 " " $CaCl_2$ in 0.85 per cent NaCl	"	Phosphate buffered " " " "	64

* Dialyzed against 1500 volumes.
‡ 4 hemagglutinating units of Lee virus employed.

calcium ions and release the virus from combination. To test this hypothesis the following experiments were carried out.

To unheated guinea pig serum was added an equal volume of Lee virus–infected allantoic fluid, and the mixture was incubated at 37°C. for 30 minutes. An aliquot was diluted in saline, and a second aliquot was diluted in 2.5 per cent sodium citrate. The dilutions were held for 15 minutes at 37°C. and the hemagglutination titers were then determined. Heated serum was treated in an identical manner.

The results of three experiments with different Lee virus preparations are shown in Table X. It will be noted that release of virus from combination with the heat labile serum component in the presence of sodium citrate was not consistent, and was of varying degree. The largest amount of virus that was released was only about 25 per cent of that added to the serum. In many experiments it was not possible to show that any virus was released from combination by citrate. In no instance in which PR8 was used was it possible to demonstrate release of virus once combination had taken place, but with mumps virus release of about 1 per cent was demonstrable.

The effect of prolonged incubation of mixtures of unheated serum and virus was investigated to determine whether this would cause more complete release of virus.

Mixtures of unheated guinea pig serum and Lee virus were heated at 56°C. for 30 minutes, and also kept at room temperature for 24 hours, and the hemagglutination titers determined before and after heating at 56°C. The mixtures were diluted in either saline or 2.5 per cent sodium citrate solutions.

The results of a typical experiment are shown in Table XI. When mixtures were heated at 56°C. for 30 minutes in order to inactivate the heat labile com-

TABLE X

Effect of Removal of Calcium Ions by Citrate on Serum Component–Virus Combination

Mixture		Incuba-	Diluent	Hemagglu-
Guinea pig serum*	Lee virus prepara- tions‡	tion at 37°C.		tination titer of mixture
	No.	*min.*		
Unheated	1	30	NaCl	0
"	"	"	2.5 per cent sodium citrate	0
56°C., 30 min.	"	"	" " " " "	1024
Unheated	2	"	NaCl	0
"	"	"	2.5 per cent sodium citrate	128
56°C., 30 min.	"	"	" " " " "	1024
Unheated	3	"	NaCl	0
"	"	"	2.5 per cent sodium citrate	1024
56°C., 30 min.	"	"	" " " " "	4096

* Undiluted.
‡ Undiluted allantoic fluid.

ponent, virus was not demonstrable even when the mixture was diluted in sodium citrate solution. However, after the mixture had remained at room temperature for 24 hours and was diluted in citrate, about 6 per cent of the virus was released; when heated at 56°C. and diluted in saline, less than 1 per cent was released; but, on dilution in citrate, more dissociation occurred and about 12 per cent of the virus was demonstrable. These results indicate that spontaneous dissociation of virus-serum component combination does not occur; that some release of virus can be accomplished by appropriate procedures; that virus is not irreversibly inactivated by the serum component.

Effect of Various Chemical Reagents on the Heat Labile Serum Component.— To obtain some idea as to the nature of the serum component, the effect of various chemical reagents was studied.

TABLE XI

Partial Dissociation of Combination between Serum Component and Virus upon Prolonged Incubation and Removal of Calcium Ions by Citrate

Mixture		Mixture held at			Mixture diluted in	Hemag-glutina-tion titer of mixture
Guinea pig serum	Virus	1	2	3		
Unheated "	Lee "	37°C., 15 min. " " "	— —	— —	NaCl 2.5 per cent sodium citrate	0 0
" "	" "	" " " " " "	56°C., 30 min. " " "	— —	NaCl 2.5 per cent sodium citrate	0 0
" "	" "	" " " " " "	25°C., 24 hrs. " " "	— —	NaCl 2.5 per cent sodium citrate	0 128
" "	" "	" " " " " "	" " " " " "	56°C., 30 min. " " "	NaCl 2.5 per cent sodium citrate	16 256
56°C., 30 min.	"	" " "	" " "	" " "	" "	2048

TABLE XII

Effect of Various Procedures on the Activity of the Heat Labile Component in Guinea Pig Serum

Guinea pig serum treated with:	Conditions of treatment	Hemagglu-tination-inhibition titer[*] of serum	Embryo infectivity score[‡]
			positive/ total
Boiled trypsin 2 mg. per cc.	37°C., 120 min.	1:64	0/4
Trypsin 2 mg. per cc.	" " "	1:32	4/4
Lithium periodate 0.1 M	" " "	1:64	0/4
30 per cent methyl alcohol	4°C., 60 min.	"	—
90 " " ethyl "	23°C., 15 min.	0	—
$(NH_4)_2SO_4$, 1/2 saturated (globulin)	23°C.	0	4/4
" , saturated (albumin)	"	0	4/4
" , globulin + albumin	—	0	4/4

[*] 4 hemagglutinating units of Lee virus employed.

[‡] 10^3 E.I.D. Lee virus used.

Fresh guinea pig serum was treated with: (1) crystalline trypsin,[2] 2 mg. per cc.; (2) 0.1 M lithium periodate; (3) saturated and half-saturated ammonium sulfate; (4) 30 per cent methyl alcohol in the cold; and (5) 90 per cent ethyl alcohol. Virus hemagglutination-inhibition and neutralization titrations were then carried out with the various serum preparations and fractions.

[2] Obtained through the courtesy of Dr. M. Kunitz, The Rockefeller Institute, Princeton, New Jersey.

The results of a number of experiments are summarized in Table XII. It is of interest that neither the precipitates obtained with one-half saturated or saturated ammonium sulfate, nor a mixture of the two, contained demonstrable serum component. Trypsin inactivated the capacity of the serum component to prevent viral infection, but caused only a twofold reduction in the hemagglutination-inhibition titer. The greater quantity of serum necessary to neutralize virus infectivity as compared with the serum dilution which inhibits hemagglutination by virus, as illustrated in Fig. 1, serves as an explanation of this apparent discrepancy. Periodate did not alter the capacity of the serum component to combine with virus, and thus inhibition of both infection and hemagglutination was obtained. It will be noted that the component was not inactivated by precipitation with 30 per cent methyl alcohol in the cold, but following precipitation with ethyl alcohol at room temperature, the activity of the component was destroyed.

Relationship of Heat Labile Serum Component to Complement.—That the heat labile component of serum might be identical to complement or one of its components appeared possible. As has been pointed out, others (1, 3, 4, 6, 7) have suggested that complement may play a rôle in the neutralization of some viruses by specific antibodies. Evidence bearing on this possibility was sought by three different procedures: (*a*) the study of serum from which single components of complement had been removed by chemical treatment; (*b*) the study of serum from which complement had been removed by an antigen-antibody precipitate; and (*c*) the study of serum from which most of the heat labile component had been removed by combination with virus.

1. Effect of "Incomplete" Complement on Lee Virus.—

Different fractions of guinea pig serum obtained through the courtesy of Dr. Michael Heidelberger, College of Physicians and Surgeons, Columbia University, were employed: (*a*) "endpiece" represented a fraction from which midpiece (C_1) had been removed as well as some of C_3, but all of C_2 and most of C_4 were present; and (*b*) "midpiece" consisted of a fraction from which endpiece (C_2) and some of C_4 had been removed, but all of C_1 and most of C_3 remained. In addition, similar endpiece and midpiece fractions were prepared from guinea pig serum in this laboratory in the following manner. Six cc. of guinea pig serum was dialyzed in a rocker against 750 cc. of phosphate buffer pH 5.4, ionic strength 0.02, for 6 hours at 4°C.; a change of buffer was made after 3 hours. The material was then centrifuged at 8,000 R.P.M. for 10 minutes. The supernate was decanted, neutralized immediately, and brought to isotonicity with NaCl. This fraction is termed "endpiece." The precipitate was broken up and well mixed with 6 cc. of phosphate buffer, and then was recentrifuged. The final precipitate was resuspended in 6 cc. 0.85 per cent NaCl containing 0.1 per cent $CaCl_2$. This fraction is termed "midpiece."

The results of an experiment with endpiece and midpiece fractions and Lee virus are presented in Table XIII. It will be noted that neither endpiece nor midpiece prevented viral infection, whereas a mixture of the two fractions neu-

tralized virus infectivity in 3 of 4 embryos. It should be emphasized that these fractions contain numerous constituents in addition to components of com-

TABLE XIII

Effect of Guinea Pig Complement Fractions on Infectivity of Lee Virus

Mixture		Incubation at 37°C.	Hemagglutination titers allantoic fluids*				Embryo infectivity score
Guinea pig serum fraction	Lee virus		A	B	C	D	
	E.I.D.	min.					*positive/total*
Whole serum	10^2	30	0	0	0	0	0/4
" " 56°C., 30 min.	"	"	>128	>128	>128	>128	4/4
"Endpiece" (C₂, C₃, C₄)	"	"	"	"	"	"	4/4
"Midpiece" (C₁, C₃, C₄)	"	"	"	"	"	"	4/4
"Endpiece + midpiece" fractions	"	"	0	0	32	0	1/4
" + " " 56°C., 30 min.	"	"	>128	>128	>128	>128	4/4

* Removed after incubation of embryos for 2 days at 35°C.

TABLE XIV

Effect of Removal of Complement by an Antigen-Antibody Precipitate on the Serum Components

Mixture				Incubation at 37°C.	Results of serum titrations			
Guinea pig serum	Streptococcus MG immune rabbit serum*	Streptococcus MG polysaccharide	NaCl		Complement titer‡	Hemagglutination-inhibition titer§	Virus neutralization¶	
							Serum dilution	Embryo infectivity score
cc.	cc.	mg.	cc.	min.	cc.			*positive/total*
0.45	0.1	0	0.35	30	<0.004	1:64	1:4	0/4
							1:8	1/4
							1:16	4/4
0.45	0.1	0.035	0	30	>0.20	1:32	1:4	4/4

* Diluted 1:2 and heated 65°C. for 30 minutes.

‡ Quantity of guinea pig serum required to hemolyze completely a 3 per cent suspension of sensitized sheep RBC.

§ 4 hemagglutinating units of Lee virus used.

¶ 10^2 E.I.D. Lee virus employed.

plement, and that a mixture of endpiece and midpiece fractions is in effect a reconstituted serum. Neither the endpiece nor the midpiece fraction alone had complementary activity, but a mixture of the two was effective in lysing sensitized sheep RBC. Results of hemagglutination-inhibition titrations with

these preparations were irregular, and could not be interpreted satisfactorily due to the fact that heat stable inhibitors were present.

2. Effect of Removing Complement with an Antigen-Antibody Precipitate on the Heat Labile Serum Component.—

To fresh guinea pig serum were added inactivated antistreptococcus MG rabbit serum and capsular polysaccharide of streptococcus MG. It is known that the polysaccharide-antibody precipitate which forms in such a mixture binds complement (22). The mixture was held at 37°C. for 30 minutes, following which it was centrifuged. The precipitate was discarded, and the supernate employed in hemagglutination-inhibition, neutralization, and complement titrations.

TABLE XV

Effect of Removal of Serum Component by Virus on Complement Titer

Mixture				Results of serum titrations			
Guinea pig serum	Lee virus allantoic fluid heated 65°C. for 30 min.	Normal allantoic fluid	Incubation at 37°C.	Complement titer*	Hemagglu-tination-inhibition titer‡	Virus neutralization§	
						Serum dilution	Embryo infectivity score
cc.	*cc.*	*cc.*	*min.*	*cc.*			*positive/total*
0.7	0	0.7	15	0.004	1:320	1:4	0/4
						1:8	2/4
						1:16	3/4
0.3	0	1.2	"	0.005	1:320	—	—
0.7	0.7	0	"	0.004	1:80	1:4	4/4
0.3	1.2	0	"	"	<1:20	—	—

* Quantity of serum which completely lysed sensitized sheep RBC.
‡ 4 hemagglutinating units of Lee virus used.
§ 10^2 E.I.D. Lee virus employed.

The results of a typical experiment are presented in Table XIV. The precipitate resulting from the interaction of streptococcus MG polysaccharide and specific antibodies against it not only removed complement but also removed the serum component which neutralized Lee virus infectivity. However, as indicated by the results of hemagglutination-inhibition titrations, all the serum component was not removed. On this basis the reduction in the concentration of the component was only twofold, whereas the reduction in complement concentration was at least 50-fold.

3. Effect of Virus-Serum Component Combination upon Complement Titer.—

One and 4 volumes, respectively, of Lee virus–infected allantoic fluid heated at 65°C. for 30 minutes were added to aliquots of guinea pig serum. Such treated virus is not infectious,

and does not cause hemagglutination. Similar mixtures of serum and normal allantoic fluid were prepared. Each mixture was held at 37°C. for 30 minutes, and then complement, hemagglutination-inhibition, and neutralization titrations were carried out.

The results of two experiments are summarized in Table XV. The concentration of complement was undiminished even when 4 volumes of virus was mixed with serum, but the concentration of the serum component, as determined by hemagglutination-inhibition, was below the amount which could be measured by this technique representing at least a 16-fold reduction. Similarly, when equal volumes of serum and virus were mixed, there was a definite reduction in the concentration of the serum component as determined both by neutralization and hemagglutination-inhibition without any reduction in the complement titer. These results indicate that the heat labile serum component is not identical to complement.

DISCUSSION

That the serum of human beings, guinea pigs, rabbits, and mice contains a thermolabile component which inactivates influenza A and B, mumps, and Newcastle disease viruses on combining with them is indicated clearly by the evidence presented. The lability of this component when stored at 4°C. and on heating, in addition to the finding that its activity is diminished by crystalline trypsin and destroyed by ethyl alcohol, suggests that it may be an unstable protein or protein complex. The serum component–virus combination is stable and dissimilar to that which occurs between heat-resistant inhibitors of viral hemagglutination present in the serum of rabbits and ferrets (18) as well as in normal allantoic fluid (19) and egg white (20). The latter inhibitors are partially or completely inacitvated by virus and the virus is then released, whereas with the heat labile serum component spontaneous release of virus does not occur. Some release of virus may be achieved by the addition of sodium citrate to the mixture which probably is a result of the removal of calcium ions necessary for combination. A further and most important difference lies in the fact that with but rare exceptions (23, 24) inactivated serum of normal animals does not neutralize the infectivity of the viruses employed in this study.

Certain properties of the heat labile serum component are similar to those of complement, and certain workers (1, 3, 4, 6, 7) have attributed their results with other viruses as due to complement activity. Because the thermolabile component is not removed with complement in like amount by an antigen-antibody precipitate, and because complement is not removed by a quantity of virus sufficient to combine with all demonstrable serum component, it is unlikely that the two are identical.

The fact that there is present in human and animal serum a thermolabile component which can combine with any of the four viruses studied, and

can neutralize the infectivity of these agents, makes it hazardous to employ unheated sera in virus neutralization tests. Differences in the manner in which sera are handled as to separation from the clot, as well as temperature and duration of storage, may markedly affect the results. As example, sera stored at 4°C. for 2 to 4 weeks will usually not contain active component, whereas sera stored for only a few days at this temperature or held for long periods at −28°C. may neutralize 10^3 to 10^4 E.I.D. of virus. Under such circumstances the results might be taken as evidence that specific neutralizing antibodies had developed when this was not the case. Likewise, the addition of unheated normal serum to heat-inactivated immune serum may yield peculiar results which may be difficult to interpret.

It may be that the serum component affects attempts to recover certain viruses from the blood. For example, only rarely has mumps virus been recovered from the blood of a patient (25), despite the evidence that viremia is present in a considerable proportion of patients. It seems probable that unless the quantity of virus present in the blood were relatively large, 10^4 to 10^5 E.I.D. per cc., the heat labile component would prevent infection of susceptible animals. Brooksby (26) has pointed out that the virus of hoof-and-mouth disease is inactivated much more rapidly in defibrinated than in citrated guinea pig blood because calcium ions are needed for inactivation. Such inactivation may be a result of combination of virus with the serum component.

SUMMARY

A labile component present in the serum of human beings, guinea pigs, and rabbits neutralizes the infectivity of mumps, Newcastle disease, influenza A and B viruses. The labile component of these sera and of mouse serum also inhibits hemagglutination of chicken RBC by these viruses. The component is inactivated by heating at 56°C. for 30 minutes and upon storage at 4°C. for periods longer than 2 weeks. The virus-neutralizing and hemagglutination-inhibiting properties result from serum component–virus combination in the presence of calcium. The combination is stable, and does not undergo spontaneous dissociation. Partial separation of virus can be brought about by heating mixtures held for 24 hours or by removal of calcium ions with sodium citrate. The labile serum component appears to be distinct from hemolytic complement.

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LACK OF IDENTITY IN NEUTRALIZING AND HEMAGGLUTINA-TION-INHIBITING ANTIBODIES AGAINST INFLUENZA VIRUSES

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Following the development of the hemagglutination-inhibition technique for the measurement of antibodies directed against influenza virus, this *in vitro* procedure largely supplanted *in vivo* neutralization techniques. It has been generally considered that the hemagglutination-inhibition and neutralization techniques measure the same antibody. Justification for this assumption appears to rest mainly on the demonstration by Hirst (1) of a fairly good correlation between serum antibody titers measured by hemagglutination-inhibition and titers measured by neutralization in mice. Some support for the concept also was provided by absorption experiments of Wiener, Henle, and Henle (2) which showed that both hemagglutination-inhibiting and neutralizing antibodies were absorbed from immune serum by concentrated influenza virus.

Some evidence has been obtained, however, which suggests that the hemagglutination-inhibition and neutralization techniques may measure different antibodies. Burnet and Beveridge (3) and Stuart-Harris and Miller (4) have demonstrated marked discrepancies in the antibody titers of sera as measured by hemagglutination-inhibition and by neutralization in mice or *in ovo*. In addition, Friedewald (5) found that absorption of antiserum with influenza virus resulted in a proportionately greater reduction in neutralizing titer than in hemagglutination-inhibiting titer.

Because of the theoretical and the practical implications of this problem, it appeared to merit further study. The results obtained in the present investigation indicate that there is an exponential linear relationship between the quantity of virus and antibody in *in ovo* neutralization; that the concentration of neutralizing antibody can be measured with considerable precision *in ovo*. It will be shown that the amounts of hemagglutination-inhibiting and neutralizing antibodies which are absorbed by a given quantity of virus are predictable; that the reactivity of these antibodies is directly related to the extent of immunization. It will be demonstrated that there are marked discrepancies in correlation between antibody titers obtained by *in vitro* and *in vivo* techniques. Moreover, it will be shown that neutralizing antibody is preferentially absorbed by a given quantity of virus. On the basis of these results it

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appears probable that hemagglutination-inhibiting and neutralizing antibodies are not identical.

Materials and Methods

Virus.—The PR8 strain of influenza A virus was used. This strain previously had been passed many times in mice and in chick embryos. The virus was cultivated in the allantoic sac of White Leghorn chick embryos which had been incubated at 39°C. for 10 to 12 days. After inoculation with approximately $10^{4.5}$ E.I.D. of virus, the eggs were incubated at 35°C. for 48 hours and then chilled at 4°C. overnight or at −20°C. for $1\frac{1}{2}$ hours before the allantoic fluids were harvested. Infected fluids used for *in ovo* neutralization experiments were diluted with 9 parts of sterile normal horse serum (previously heated at 56°C. for 30 minutes) and stored in nitrocellulose tubes at −70°C. Infected fluids used as a source of virus for serum absorption experiments were sterile pooled fluids from groups of 60 to 100 eggs and were sealed in glass ampoules and stored at −70°C. until used.

Virus Titrations.—Hemagglutination titrations were done in a manner similar to the technique described by Hirst (1). Serial twofold dilutions of allantoic fluid were made in saline buffered at pH 7.2. To 0.4 cc. of each dilution was added 0.4 cc. of a 1 per cent suspension of chicken RBC in buffered saline. Readings were made after the tubes had stood for 1 hour at room temperature. The end point was taken as the highest dilution which gave a definite 2+ pattern of RBC agglutination.

Virus infectivity titrations in chick embryos were done by the intra-allantoic technique in 10 to 12 day old embryos. Serial tenfold dilutions of allantoic fluids were made in sterile broth containing 10 per cent normal horse serum. A group of embryos was inoculated with each dilution and each embryo received 0.2 cc. Following incubation at 35°C. for 48 hours, the allantoic fluids were harvested individually and tested by the hemagglutination technique. The 50 per cent infectivity end point was calculated according to the method of Reed and Muench (6). Virus infectivity titrations in mice were carried out exactly as described previously (7).

Immune Serum.—Young adult rabbits were given an intravenous injection of 10 cc. of undiluted infected allantoic fluid. The rabbits were bled from the heart 2 weeks later. Prolonged immunization was carried out with a number of rabbits. Following the first bleeding they were given an intraperitoneal injection of 10 cc. of infected allantoic fluid. At 2 week intervals thereafter they were bled and reinjected with 10 cc. of allantoic fluid intraperitoneally until 3 or 4 injections had been given. The serum was stored frozen at −20°C. Human convalescent serum obtained from patients hospitalized with influenza A during the winter of 1943–44 was stored at 4°C. without preservatives. Before use in hemagglutination-inhibition or *in ovo* neutralization tests all serum was heated at 56°C. for 30 minutes.

Antibody Titrations.—Hemagglutination-inhibition titrations were carried out in a manner similar to that described by Hirst (1). Serial twofold dilutions of inactivated serum were made in saline. To 0.2 cc. of each dilution was added 0.2 cc. of infected allantoic fluid diluted so as to produce a final concentration of 8 hemagglutination units of virus in each tube. To each mixture was added 0.4 cc. of a 1 per cent suspension of chicken RBC. Readings were made after 1 hour at room temperature and the end point was taken as the highest dilution of serum which completely inhibited RBC agglutination. Titrations were always carried out in parallel with the same reagents when it was desirable to compare the antibody levels of two or more sera.

In ovo neutralization titrations were carried out by the intra-allantoic technique in 10 to 12 day old embryos. Serial twofold dilutions of inactivated serum were made in 10 per cent horse serum broth. To 0.5 cc. of each serum dilution was added 0.5 cc. of infected allantoic

fluid diluted in 10 per cent horse serum broth so as to contain the desired number of E.I.D. of virus. In routine titrations the mixtures were held at 4°C. for 30 minutes and each mixture was then inoculated into a group of 4 embryos; each embryo received 0.2 cc. Allantoic fluid was harvested from each embryo after 48 hours' incubation at 35°C. and tested by the hemagglutination technique. The serum dilution end points were calculated by the 50 per cent end point method of Reed and Muench (6). Each neutralization test was controlled by a titration of the virus done at the same time. The quantity of virus actually used in the neutralization test was determined by subtracting the final dilution of virus in the serum-virus mixtures from the virus dilution end point of the control virus titration. Neutralization tests in mice were carried out exactly as described previously (7).

Concentration of Virus.—Virus for use in absorption experiments with immune serum was concentrated from PR8-infected allantoic fluid by sedimentation in a high speed vacuum centrifuge (8). Large sterile pools of allantoic fluid with a hemagglutination titer of about 1:1024 were prepared for this purpose. When it was desirable to compare results obtained after absorption of aliquots of a serum or of aliquots of two or more sera, a single pool of allantoic fluid was used as a source of virus.

The allantoic fluid was distributed in nitrocellulose tubes (9 cc. per tube) and subjected to a mean gravitational field of 30,000 g. for 40 minutes or 37,900 g. for 30 minutes. After centrifugation the supernatant fluid, except for about 0.4 cc. above the sediment, was removed from each tube with a fine tipped pipette. The sediments were resuspended in the remaining supernate by repeated aspiration and ejection with a fine tipped pipette. Sufficient sterile saline, buffered at pH 7.2, was added to bring the volume to about $\frac{1}{6}$ the original and the fluid was again subjected to centrifugation as described above. After the second centrifugation all but about 0.3 to 0.5 cc. of the supernatant fluid was removed and sterile buffered saline was added to the sedimented material to bring the volume to the desired level, usually 1.0 cc. The sediment was again resuspended by repeated aspiration and ejection with a fine tipped pipette. The results of direct tests showed that each concentrated virus preparation contained at least 99 per cent of the virus originally present in the infected allantoic fluid pool.

Absorption of Antibody.—Immune serum was heated at 56°C. for 30 minutes. Serum which had been stored for a time sufficient to allow the development of a white amorphous precipitate was centrifuged and the clear supernatant serum was removed and used in the experiment. In general, 1.0 cc. of serum was added to an equal volume of concentrated virus and thoroughly mixed. At the same time 1.0 cc. of serum was mixed with 1.0 cc. of sterile saline as a control. The mixtures were held overnight at 4°C. and were then centrifuged at 4°C. in the high speed vacuum centrifuge in a gravitational field of 37,900 g. for 30 minutes. After centrifugation the supernatant serum, except for about 0.3 to 0.4 cc. above the sediment, was carefully withdrawn. The supernates were heated at 65°C. for 40 minutes to eliminate any unsedimented virus which might remain and their antibody content was measured by means of the hemagglutination-inhibition and the *in ovo* neutralization techniques. The absorbed serum and the unabsorbed saline control were always compared for antibody titer in the same test using the same reagents. The amount of antibody absorbed was calculated from the difference in titers shown by the absorbed serum and the unabsorbed control in the same test.

<div align="center">EXPERIMENTAL</div>

Quantitative Relationship between Virus and Antibody in in Ovo Neutralization.—It has been shown (7) previously that a linear relationship exists between the quantity of influenza A virus neutralized and the quantity of serum employed in neutralization experiments in mice. Such a relationship also has been demon-

strated (9) *in ovo* with certain strains of influenza virus. This quantitative relationship between immune serum and virus *in ovo* was studied with the PR8 strain which was employed in the present investigation in order to obtain information as to the reproducibility of neutralization titers determined *in ovo* and the effect of varying amounts of virus upon such titers.

Dilutions of allantoic fluid infected with the PR8 strain ranging from 10^{-3} to 10^{-7} were each mixed with a number of twofold dilutions of anti-PR8 rabbit serum. Each mixture was inoculated intra-allantoically into a group of chick embryos. The details of the procedure employed and of the calculation of the quantity of virus used as well as the serum dilution end point are given above.

The results obtained in a series of neutralization experiments *in ovo* with two homologous antisera are presented graphically in Fig. 1. The logarithm of each serum dilution end point is plotted against the logarithm of the quantity of virus neutralized. Parallel straight lines which appeared best to fit the end points obtained with each serum were drawn. The slope of the lines shown in Fig. 1 is approximately 4.7, indicating that a change of 5.7 log units in the quantity of virus employed causes a change of only 1.0 log unit in the serum dilution end point. Similar surprisingly steep slopes in *in ovo* neutralization experiments with other strains of influenza A virus and either human or ferret immune serum have been reported previously by Burnet (9). It should be emphasized that in comparable experiments in mice the slope of the neutralization line was found (7) to be 1.44. Moreover, in hemagglutination-inhibition experiments *in vitro* the linear relationship between virus and antibody has been shown (1) to have a slope of 1.0.

Experiments similar to those illustrated in Fig. 1 also were carried out with the Lee strain of influenza B virus and homologous immune rabbit serum. Four different quantities of virus were employed. The slope of the neutralization line obtained was almost identical to that found with the PR8 strain.

It can be seen from the slope of the line drawn through the neutralization end points in Fig. 1 that antiserum rapidly loses efficiency in its ability to neutralize virus as it is diluted. It was found that when neutralization tests were carried out with dilutions of virus and a constant quantity of serum the results varied markedly within individual groups of embryos which resulted in the spreading of both positive and negative allantoic fluids over a number of virus dilution–serum mixtures. As a consequence, the reproducibility of neutralization end points obtained in this manner was relatively poor. With the 22 end points shown in Fig. 1 the mean deviation from the lines is ± 0.61 log unit along the *y* axis (vertically). On the other hand, when titrations were carried out with a constant virus concentration and dilutions of serum, the results were much sharper and far more reproducible end points were obtained. With the 22 end points shown the mean deviation from the lines is ± 0.14 log unit

along the x axis (horizontally). It is evident, because of the steep slope of the *in ovo* neutralization line, that in titrations carried out by this method the end points are but little affected by considerable variations in the amount of virus employed. A change of 1.0 log unit in the quantity of virus used results in a shift in the serum dilution end point of only 0.21 log unit.

On the basis of the results obtained in this study on the quantitative relationship between the quantities of serum and virus in neutralization experiments
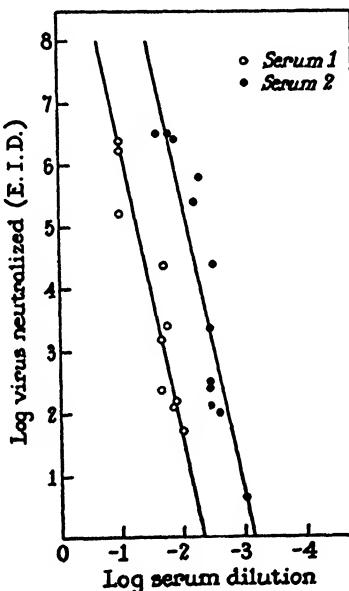
FIG. 1. Linear relationship between the quantity of virus and the quantity of immune serum in *in ovo* neutralization titrations with influenza virus (PR8). The slope of the lines shown is 4.7, indicating that a tenfold change in serum dilution end point (x axis) corresponds to a 500,000-fold change in the amount of virus neutralized (y axis).

in ovo, the constant virus–serum dilution technique was adopted for use in all further experiments.

In the experiments described above virus–serum mixtures were held at 4°C. for 30 minutes before inoculation of chick embryos. Other experiments in which virus–serum mixtures were held for 24 hours at 4°C. were also carried out. The same two rabbit antisera were used; 3 end points were determined with one and 4 with the other. It was found that holding mixtures for 24 hours did not affect the serum dilution end point when large amounts of virus were used; *i.e.*, in mixtures in which the concentrations of virus and serum were high. However, as the quantity of virus was reduced, the serum dilution end point tended to increase; this was especially evident in mixtures in which the

concentrations of virus and serum were low. The slope of the *in ovo* neutralization line obtained with mixtures held 24 hours at 4°C. was of the order of 3. A slight decrease in the slope of the neutralization line under similar conditions with ferret immune serum was reported by Burnet (9).

Correlation between Hemagglutination-Inhibition and in Ovo Neutralization Titers.—Previous workers (3, 4) have pointed out discrepancies between the results of the neutralization and hemagglutination-inhibition techniques as means of determining the concentration of antibodies against influenza viruses. A study of the correlation between antibody titers against the PR8 strain as measured by *in ovo* neutralization and hemagglutination-inhibition was undertaken.

Preliminary titrations of both neutralizing and hemagglutination-inhibiting antibodies were carried out with a number of anti-PR8 rabbit sera and convalescent sera from patients with influenza A. The techniques employed were identical to those described above; in hemagglutination-inhibition titrations a final concentration of 8 units of PR8 was used, in neutralization titrations 10^3 E.I.D. of virus was used. When two sera were found which showed approximately equal hemagglutination-inhibition titers but different *in ovo* neutralization titers, they were more closely studied in parallel titrations of both types.

Forty-one immune rabbit sera and 11 convalescent human sera were studied in this manner. Within this group there were 11 pairs of rabbit sera which did not show as much as a twofold difference in the hemagglutination-inhibition titers but did show, between themselves, differences in *in ovo* neutralization titers which ranged from four- to 13-fold. Similarly, three pairs of convalescent human sera were found which showed no difference in hemagglutination-inhibition titers, but had six- to ninefold differences in neutralizing titers.

One such pair of anti-PR8 rabbit sera was selected for intensive study. With these sera (sera 1 and 2) 8 hemagglutination-inhibition titrations were carried out in parallel. The titrations failed to reveal any consistent difference in the hemagglutination-inhibition titers of the two sera. The geometric mean of the 8 titrations was 1:1024 with serum 1 and 1:940 with serum 2. The results obtained when the two sera were compared by *in ovo* neutralization using a wide range of virus concentrations are presented in Fig. 1. It can be seen that, although the sera did not differ significantly in hemagglutination-inhibition titer, serum 2 had a mean neutralizing titer sixfold higher than serum 1 over the entire range studied.

It is evident that the correlation between hemagglutination-inhibition titers and *in ovo* neutralization titers in the group of sera studied was poor. The poor correlation raised the possibility that the hemagglutination-inhibition and neutralization techniques measure different antibodies directed against influenza virus. Further experiments were carried out to test this hypothesis.

Absorption of Specific Antibody with Influenza Virus.—In order to test more fully the possibility that hemagglutination-inhibition and *in ovo* neutralization

measure different antibodies, it was decided to utilize the classical method of antibody absorption.

Details of the procedure used are given above. For the purpose of controlling the procedure anti-PR8 sera were absorbed with a quantity of heterologous Lee virus equal to that used with PR8 virus in specific antibody absorptions. Several experiments demonstrated that absorption with Lee virus did not give results significantly different from those obtained when immune sera were mixed with saline and carried through all steps of the procedure. In subsequent experiments such saline controls were used for comparison with PR8-absorbed sera. Experiment showed that the amount of centrifugation employed as routine in the absorption procedure caused sedimentation of at least 99.9 per cent of the virus added to a 1:2 dilution of rabbit serum in saline and that heating the supernate of such a mixture at 65°C. for 40 minutes eliminated any demonstrable virus remaining in the supernate.

Preliminary experiments showed that both the hemagglutination-inhibiting and the neutralizing titers of anti-PR8 serum were reduced by absorption with PR8 virus; that the neutralizing titer was reduced to a considerably greater extent than the hemagglutination-inhibiting titer; that the neutralizing titer could be reduced to a very low level even though the serum still retained a high titer of hemagglutination-inhibiting antibodies. A typical experiment illustrating these points was carried out as follows:—

Aliquots of an anti-PR8 serum were absorbed with virus concentrated from 5, 10, and 20 cc., respectively, of PR8-infected allantoic fluid. After absorption the hemagglutination-inhibition and *in ovo* neutralization titers of each aliquot were determined in parallel with those of the unabsorbed saline control serum.

The results obtained are presented in Table I. It can be seen that, as increasing quantities of virus were used for absorption, there was a regular and a progressive reduction in the antibody titers of the serum as measured by both hemagglutination-inhibition and *in ovo* neutralization. It is evident, however, that there was a greater reduction in neutralizing titer than in hemagglutination-inhibition titer and that the discrepancy between the two titers increased as more and more antibody was removed until, with the use of virus from 20 cc. of allantoic fluid, the *in ovo* neutralization titer was reduced 76-fold while the hemagglutination-inhibition titer was decreased by only eight-fold. This represents a reduction in neutralizing titer 9.5 times greater than in hemagglutination-inhibiting titer.

Comparison of the Sensitivity of the Hemagglutination-Inhibition and the in Ovo Neutralization Techniques in Measuring Known. Differences in Antibody Concentration.— Because hemagglutination-inhibition and *in ovo* neutralization are such markedly different procedures, one carried out *in vitro* and the other *in vivo*, it was considered possible that the apparently greater absorption of neutralizing antibody could be due to differences in the sensitivity of the two techniques in measuring changes in antibody concentration. Experiments

were carried out to compare the two procedures in the measurement of known changes in antibody concentration.

Anti-PR8 rabbit serum was diluted 1:4, 1:16, and 1:64 with normal rabbit serum. Aliquots of these dilutions, together with an aliquot of undiluted antiserum, were each further diluted 1:2 in saline, centrifuged at 37,900 g. for 30 minutes, and the supernatant serum heated at 65°C. for 40 minutes in order to reproduce the conditions of the absorption experiments. The antibody concentration of each of the specimens was then measured 3 separate times by the hemagglutination-inhibition technique and once by the *in ovo* neutralization technique. All titrations were carried out in parallel.

TABLE I

Absorption of Specific Antibodies with Influenza Virus (PR8)

Amount of virus	Antibody				Ratio of change in titers
	Hemagglutination-inhibition		Neutralization *in ovo*		Neutralization
					Hemagglutination-inhibition
	Titer	Decrease in titer	Titer	Decrease in titer	
cc. all. fl. per cc. serum*		fold		fold	
0	8192	—	835	—	
5	4096	2	354	2.4	1.2
10	2048	4	64	13	3.3
20	1024	8	11	76	9.5

* Anti-PR8 rabbit serum.

The results of one experiment are presented in Table II. The titers found with each diluted serum specimen are recorded and it can be seen that both the hemagglutination-inhibition and *in ovo* neutralization titers all fall within less than 0.2 log unit of the theoretical titers computed on the basis of dilution. The reproducibility of the serum dilution end points obtained by either technique is evident when the titers are expressed in terms of the undiluted serum. As is seen, the deviation of such computed end points from the geometric mean was ± 0.07 log unit with hemagglutination-inhibition and ± 0.03 log unit with *in ovo* neutralization.

In Fig. 2 the results of two such experiments are presented graphically. The logarithm of the hemagglutination-inhibition titers of the diluted sera is plotted against the logarithm of the *in ovo* neutralization titers. Inasmuch as the experiments were carried out with two different immune rabbit sera which had different antibody levels, the two sets of experimental points do not fall along the same line. Lines with a slope of 45° which best fit the points obtained with each serum have been drawn. These lines represent the theoretical end points which should be obtained with known changes in antibody concentration. It can be seen that the experimental end points fall very close to the

TABLE II

Measurement of Known Changes in Antibody Concentration by Hemagglutination-Inhibition and in Ovo Neutralization

| Dilution of immune serum* in normal serum | Antibody | | | | | |
| | Hemagglutination-inhibition | | | Neutralization *in ovo* | | |
	Titer found	Titer computed to undiluted serum	Deviation from geometric mean	Titer found	Titer computed to undiluted serum	Deviation from geometric mean
	log	*log*	*log*	*log*	*log*	*log*
0	−3.91	−3.91	+0.07	−3.10	−3.10	0.0
1:4	−3.31	−3.91	+0.07	−2.48	−3.08	−0.02
1:16	−2.51	−3.71	−0.13	−1.95	−3.15	+0.05
1:64	−2.01	−3.82	−0.02	−1.26	−3.07	−0.03
Geometric mean....		−3.84	±0.07		−3.10	±0.03

*Anti-PR8 rabbit serum.
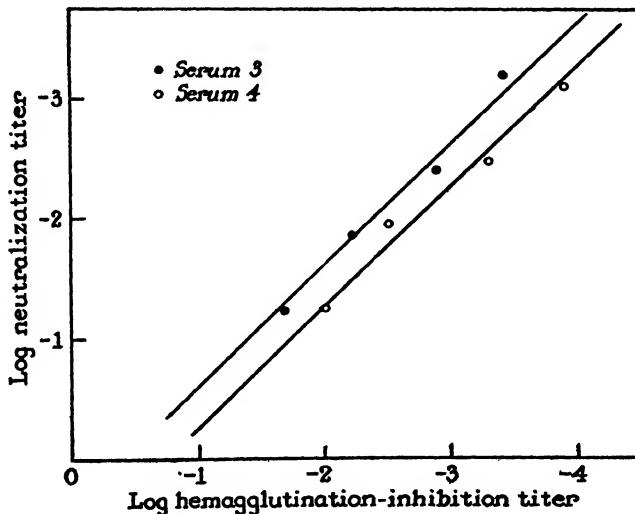
FIG. 2. Correlation between experimentally determined and theoretical end points measured *in vitro* and *in vivo* with known decrements in antibody concentration. The slope of the lines shown, which correspond to the thoretical end points, is 1.0.

appropriate 45° line, indicating that both methods of antibody measurement are sufficiently sensitive to detect the changes which were produced in antibody concentration by serial fourfold dilution of the sera. It is, therefore, evident that the disproportionate reduction in neutralizing antibody titer produced

by absorption of anti-PR8 serum with PR8 virus cannot be attributed to a difference in the sensitivity with which the *in vivo* and *in vitro* techniques measure changes in antibody concentration.

Absorption of Various Immune Sera with Influenza Virus.—Numerous experiments were carried out in which specific antibodies were absorbed from immune serum with concentrated PR8 virus. Both convalescent human and immune rabbit sera were used and various virus-antibody ratios were employed. The details of the procedure and a description of the controls used are given above.
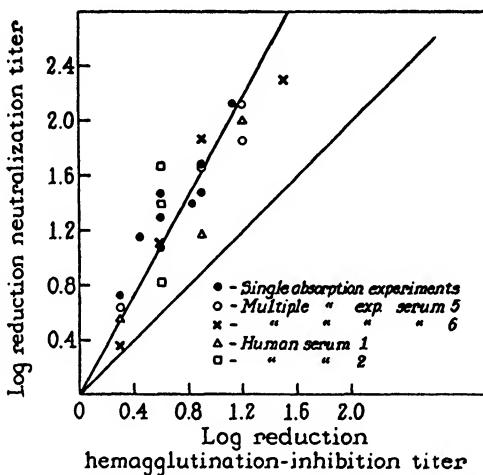
FIG. 3. Relation between quantity of neutralizing antibody and hemagglutination-inhibiting antibody absorbed from immune sera by influenza virus (PR8). Preferential absorption of neutralizing antibody is indicated by deviation of experimental line from theoretical line.

In Fig. 3 the results of 23 serum absorption experiments are presented graphically. Of the 23 experimentally determined points, 14 represent values derived from the geometric means of end points determined in two or more *in ovo* and *in vitro* titrations. The logarithm of the reduction in the hemagglutination-inhibition titer is plotted against the logarithm of the reduction in the neutralization titer produced by absorption of immune serum with PR8 virus. A straight line which appeared best to fit the experimental points was drawn. In addition, a line with a slope of 45° was drawn. This line indicates the values which, in theory, should have been obtained if the two techniques measured the same antibody.

It is evident that the experimental points deviate systematically from the theoretical values, indicating that consistently more neutralizing antibody was absorbed than hemagglutination-inhibiting antibody. It can be seen that as

progressively greater amounts of antibody were removed, the deviation from the 45° line becomes wider and wider. Preferential absorption of neutralizing antibody in like amount was demonstrated in neutralization titrations in mice with some of the absorbed sera.

Two experiments were carried out in which aliquots of an immune rabbit serum were absorbed with increasing quantities of virus. A different antiserum was used in each experiment. In the first, aliquots of serum 5 were absorbed with PR8 virus concentrated from 10, 20, 30, and 35 cc., respectively, of infected allantoic fluid. In the second, aliquots of serum 6 were absorbed with virus from 5, 10, 20, and 30 cc., respectively, of allantoic fluid.

The reductions in antibody titers found in both experiments are indicated by distinctive symbols in Fig. 3. It should be pointed out that as increasing quantities of virus were used to absorb antibodies from a constant quantity of antiserum, *i.e.* as the virus-antibody ratio was increased, larger and larger amounts of both antibodies were removed, and that the selective absorption of neutralizing antibody became progressively more evident. In view of the results obtained with rabbit antisera, it was considered of importance to determine whether similar disproportionate reductions in neutralizing antibody would be produced by virus absorption of the serum of patients convalescent from influenza A.

Two convalescent human sera were used. The procedure employed was identical with that used for absorption of antibodies from rabbit serum. Absorption of each serum was carried out with three different quantities of virus.

The reductions in hemagglutination-inhibiting and neutralizing titers produced by absorption of convalescent human sera are shown in Fig. 3. It can be seen that the experimental points do not deviate markedly from the line shown and indicate that the results obtained with human sera were closely similar to those found with rabbit sera.

Relationship between the Quantity of Virus Employed and the Amount of Antibody Absorbed.—Antigen-antibody reactions have been extensively studied in recent years by means of the quantitative techniques devised and perfected by Heidelberger and Kendall (10, 11). They and other investigators have obtained much information concerning the course of reactions which occur in several protein-antiprotein and polysaccharide-antipolysaccharide systems. At the present time quantitative measurements, comparable to those employed in studies on precipitating systems, are not applicable to virus-antivirus systems unless marked purification and concentration of the virus are obtained and hyperimmune serum of very high potency is employed (12). Moreover, in the present study the objective was to determine the amounts of neutralizing and hemagglutination-inhibiting antibodies which react with influenza virus. To measure either antibody it was necessary to employ biological methods and to express results in relative rather than in absolute units. However, the
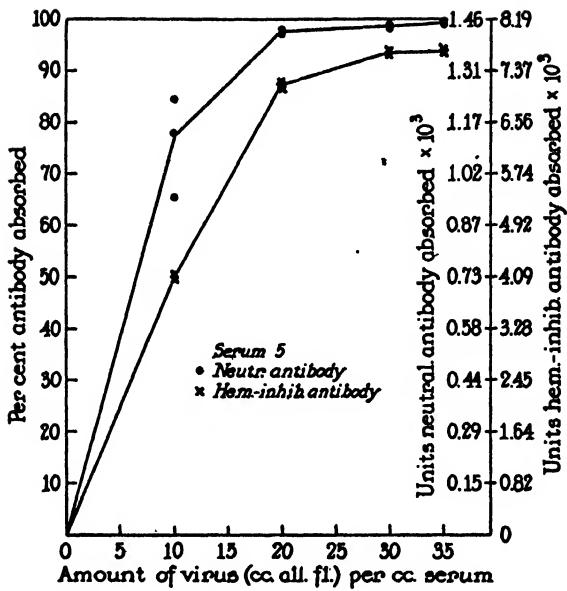
FIG. 4
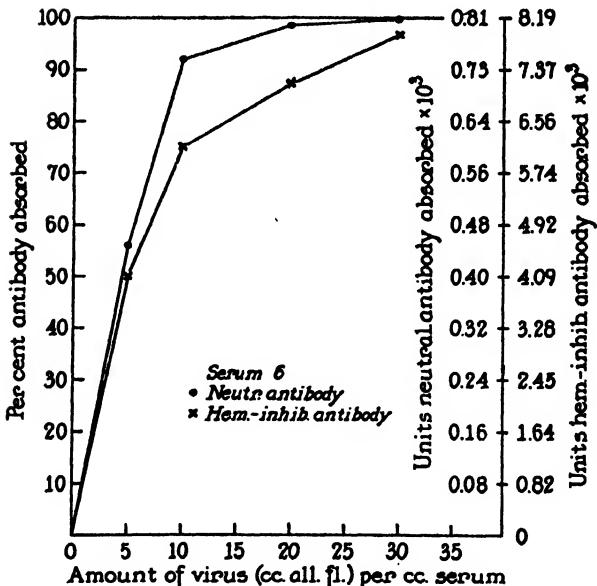
FIG. 5

FIGS. 4 and 5. Extent of absorption of neutralizing antibody and hemagglutination-inhibiting antibody from immune rabbit serum relative to the quantity of influenza virus (PR8) employed. In Fig. 4 the curves are drawn through the geometric means of the experimentally determined points.

reproducibility and apparent predictability of the results seemed to justify an analysis of the data in a manner similar to that used by Heidelberger and Kendall (10) with precipitating systems.

The relationship between the quantity of virus used and amount of both antibodies absorbed was studied in experiments with two different anti-PR8 rabbit sera (sera 5 and 6). The quantities of virus used with each serum are given in a preceding section and the reductions in antibody titers found after absorption of each serum are shown in Fig. 3. Titration end points were determined at least twice in experiments with serum 5.

In Figs. 4 and 5 the results obtained are presented graphically. The amount of virus used is plotted againt the per cent as well as the number of units of antibody absorbed. It can be seen that the curves obtained with both hemagglutination-inhibiting and neutralizing antibody resemble those obtained with precipitin systems by other workers (10). It should be noted that throughout the course of the reaction the amount of hemagglutination-inhibiting antibody absorbed differed from the amount of neutralizing antibody absorbed. The extent of the difference reached values of definite significance when the larger quantities of virus were used. It can be seen that virus from only 16 cc. of allantoic fluid was required to absorb 90 per cent of the neutralizing antibody from 1 cc. of serum 5, whereas virus from approximately 25 cc. was necessary for absorption of 90 per cent of the hemagglutination-inhibiting antibody. Comparable figures for serum 6 show an even greater difference; 10 and 25 cc. were required to absorb 90 per cent of neutralizing and hemagglutination-inhibiting antibodies, respectively.

With precipitin systems it has been clearly established that in the zone of antibody excess a straight line is obtained when the ratio of antibody to antigen in the precipitate is plotted against the antigen added (10). In the present study, when the ratio of absorbed antibody to virus was plotted against virus added, a straight line also was obtained in the region of antibody excess. This relationship with serum 5 and serum 6 is shown in Figs. 6 and 7. For the purposes of this analysis, the neutralizing titers of the sera were multiplied by the factor necessary to make the titers numerically equal to the hemagglutination-inhibiting titers in order to allow comparison of the slopes of the lines obtained.

It should be emphasized that a straight line is obtained only in the region of antibody excess. The points obtained after addition of large quantities of virus (*i.e.*, 30 cc. in Fig. 7) represent end points in the region of antigen excess and, as a consequence, deviate from the line in the manner noted with precipitin systems (10). It can be seen that with each serum the slope of the line relating neutralizing antibody and virus differs from that for hemagglutination-inhibiting antibody. The slopes of the lines and the intercepts on the *y* axis are also different for the two sera as might be anticipated from the findings with other antigen-antibody systems (10).

Predictability of Specific Antibody Absorption with Influenza Virus.—Heidel-

berger and Kendall (10) derived a theoretical equation based on mass law considerations to express the relation between the amount of antigen added throughout the region of antibody excess. They found this equation to be applicable to several precipitin systems and by means of it were able to predict, with con-
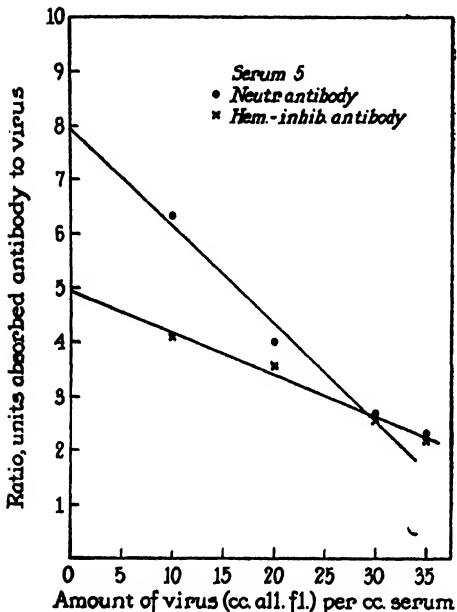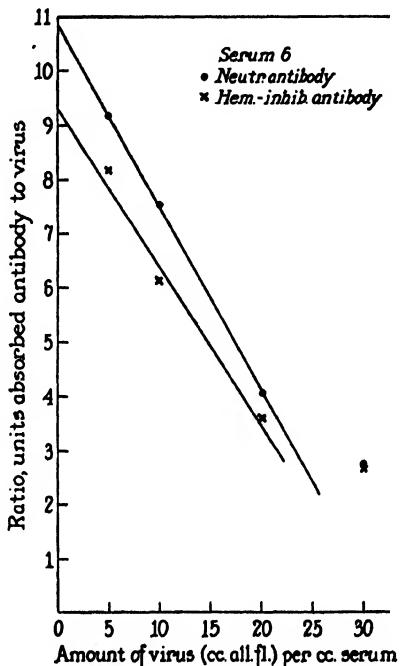
FIGS. 6 and 7. Linear relationship between the quantity of influenza virus (PR8) employed and the ratio of the amount of antibody absorbed to the amount of virus used. Experimental points in the region of antibody excess are connected by straight lines. In Fig. 6 each point was calculated from the geometric mean of two or more titrations.

siderable precision, the amount of antibody precipitated with various quantities of antigen.

The equation, $N/S = 2R - \dfrac{R^2}{A}S$, which was employed by Heidelberger and Kendall (10) for calculation of the amount of antibody nitrogen precipitated, is readily altered merely by substitution of symbols so as to be applicable to the results obtained in the present study. In the form, $Ab/V = 2R - \dfrac{R^2}{A}V$, in which Ab = units of antibody absorbed, V = amount of virus used, and A = units of antibody present in the unabsorbed serum, their equation can be used to calculate the amount of antibody absorbed when $2R$, the ratio of absorbed antibody to virus (i.e., the intercept on the y axis as shown in Figs. 6 and 7) is known. With this equation the necessary computations were carried out for the quantities of virus used with sera 5 and 6.

In Table III the calculated results for both antibodies are compared with those found experimentally. The deviations of the calculated from the experimental results are shown. It is evident that the results indicate that absorption of either antibody with PR8 virus is predictable over a considerable range in the region of antibody excess.

Increase in Reactivity of Antibodies with Prolonged Immunization.—The experiments on absorption of antibodies with PR8 virus described in preceding sections were carried out with antisera collected from rabbits after a single injection of virus. Exploratory absorption experiments revealed that anti-

TABLE III

Predictability of Absorption of Specific Antibodies with Influenza Virus

Serum No.	Absorbed with virus	Antibody					
		Neutralization units absorbed			Hemagglutination-inhibition units absorbed		
		Found	Calculated*	Deviation	Found	Calculated*	Deviation
	cc. all. fl. per cc. serum			*per cent*			*per cent*
4	10	1125	1182	+5	4096	4200	+3
4	20	1424	1455	+2	7168	6900	−4
4	30	1435	1150	−20	7680	8100	+5
5	5	451	448	−1	4096	4096	−2
5	10	741	705	−5	6144	6680	+9
5	20	794	715	−10	7168	8040	+12
Mean deviation............................				±7.2			±5.8

* Computed with the equation, $Ab/V = 2R - \dfrac{R^2}{A} V$.

PR8 antibodies in sera collected after multiple virus injections behaved in a somewhat different manner. Experiments were carried out to determine the extent of this difference.

An anti-PR8 serum (serum 7) was obtained from a rabbit after one intravenous and two intraperitoneal injections of PR8-infected allantoic fluid given at intervals of 2 weeks. Details of the immunization procedure are given above. Aliquots of the serum were absorbed with virus concentrated from 11, 16, 20, and 30 cc., respectively, of PR8-infected allantoic fluid. After absorption the hemagglutination-inhibition and *in ovo* neutralization titers of each aliquot were determined in parallel with those of the unabsorbed saline control serum. All titration end points were determined at least twice on different days.

The results obtained are presented in Table IV. It can be seen that the discrepancies between the decrease in hemagglutination-inhibition titer and the decrease in neutralization titer found in this experiment were much smaller

than those found in experiments with antisera obtained after a single injection of virus. It will be noted, too, that the extent to which neutralizing antibody was absorbed by a given amount of virus did not differ markedly from that found in experiments described above. However, absorption of hemagglutination-inhibiting antibody was much more complete and resulted in much lower titers than were attained previously with the same amount of virus.

TABLE IV

Absorption of Specific Antibodies after Prolonged Immunization with Influenza Virus(PR8)

Amount of virus	Antibody				
	Hemagglutination-inhibition		Neutralization *in ovo*		Ratio of change in titers
					Neutralization
	Titer	Decrease in titer	Titer	Decrease in titer	Hemagglutination-inhibition
*cc. all. fl. per cc. serum**		*fold*		*fold*	
0	5150	—	1580	—	—
11	1840	2.8	449	3.5	1.3
16	456	11.3	61	26	2.3
20	322	16	64	25	1.6
30	40	128	5	316	2.5

* Anti-PR8 rabbit serum 7.

In Fig. 8 the results obtained after absorption of serum 7 are shown graphically. It can be seen that the general shape of the curve representing absorption of neutralizing antibody appears quite similar to those shown in Figs. 4 and 5 (sera 5 and 6). However, the curve showing absorption of hemagglutination-inhibiting antibody closely approximates that for neutralizing antibody in both shape and height as was not the case with sera 5 and 6.

A straight line relationship also was obtained for both antibodies with serum 7 when the ratio of absorbed antibody to virus was plotted against the amount of virus employed. These relationships are shown in Fig. 9. It is apparent that the points representing the ratios obtained with the two antibodies fall very close together in contrast to those obtained with sera 5 and 6 as illustrated in Figs. 6 and 7.

Heidelberger and Kendall have shown previously (13) progressive changes in the combining characteristics of antibody from individual rabbits after successive courses of immunization. They found with precipitin systems that in the region of antibody excess a given amount of antibody combined with less antigen after each course of immunization. This resulted in precipitates with a higher ratio of antibody to antigen after each successive immunization. They interpreted their findings as indicating that on continued immunization antibody was formed which was progressively more reactive with antigen.
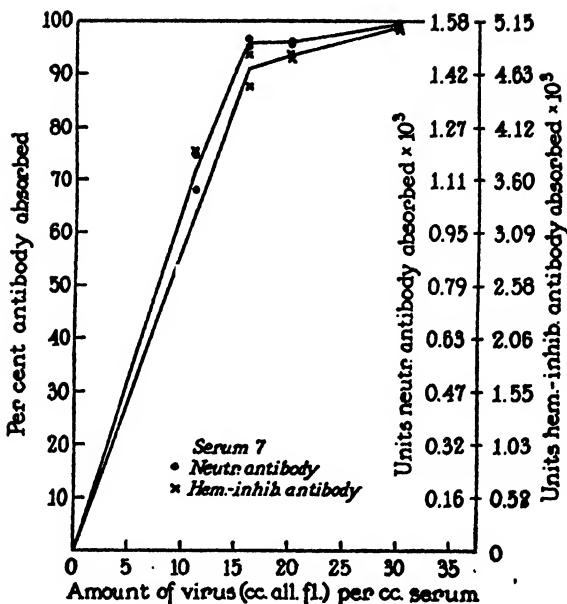
FIG. 8. Effect of prolonged immunization upon the extent of absorption of neutralizing antibody and hemagglutination-inhibiting antibody relative to the quantity of influenza virus (PR8) employed. Curves are drawn through the geometric means of the experimentally determined points.
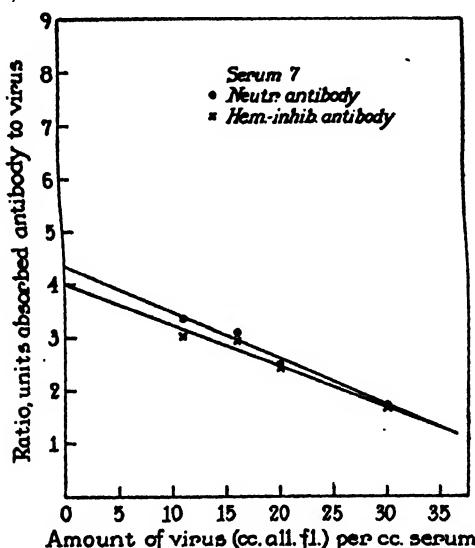
FIG. 9. Effect of prolonged immunization upon the ratio of absorbed antibodies to virus relative to quantity of influenza virus (PR8) employed. Each point was calculated from the geometric mean of two titrations.

It seemed possible that the results obtained in the experiments with serum 7 might be due to similar changes in the reactivity of one or both antibodies following continued immunization with PR8 virus. An experiment was carried out to test this possibility. A comparison was made of results found after absorption of sera obtained early and late in the course of immunization of the same rabbit.

Serum was obtained after one and after four injections of PR8 virus during the course of immunization of a rabbit. Aliquots of each serum were absorbed with virus concentrated from 10 and 20 cc., respectively, of PR8-infected allantoic fluid. After absorption the hemagglutination-inhibition and *in ovo* neutralization titers of each aliquot were determined in parallel with those of the unabsorbed saline control serum. All titration end points were determined at least twice on different days.

TABLE V

Comparison of the Absorption of Specific Antibodies from Early and Late Immune Serum of the Same Rabbit

Immune serum obtained after	Amount of virus	Antibody				Ratio of change in titers Neutralization / Hemagglutination-inhibition
		Hemagglutination-inhibition		Neutralization *in ovo*		
		Titer	Decrease in titer	Titer	Decrease in titer	
	cc. all. fl. per cc. serum		*fold*		*fold*	
1 injection of virus	0	4096	—	376	—	—
	10	2048	2	70	5.4	2.7
	20	1450	2.8	26	14.5	5.2
4 injections of virus	0	5790	—	1305	—	—
	10	1024	5.7	155	8.4	1.5
	20	64	90.4	6 or less	21.7 or more	2.4 or more

The results are presented in Table V and graphically in Fig. 10. In Table V it can be seen that even though the hemagglutination-inhibition titers of the 2 unabsorbed sera were similar, those found after absorption with equal amounts of virus were very different. As example, the titer of the early serum decreased only 2.8-fold on absorption with 20 cc. of virus, whereas the titer of the late serum was reduced 90.4-fold on absorption with an equal amount of virus. Similarly, the neutralizing titer of the late serum was reduced considerably more than that of the early serum even though the titer of the unabsorbed late serum was more than threefold higher than that of the early serum.

It should be pointed out that, as is shown in Table V, continued immunization frequently caused an increase in the neutralization titer without causing an increase in the hemagglutination titer. Among 8 rabbits given more than

one injection of virus, 5 showed fourfold rises in neutralizing titer associated with either no change or a two- to fourfold decrease in hemagglutination-inhibition titer.

When the ratio of units of absorbed antibody to virus added is plotted against virus added, after appropriate adjustment of values to correct for differences in antibody levels, the changes which have occurred on continued immunization are apparent. In Fig. 10 it can be seen that the ratio of ab-
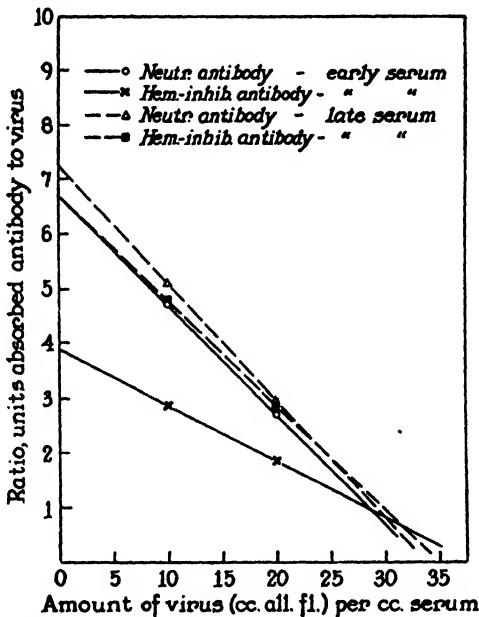
FIG. 10. Relative reactivity of neutralizing and hemagglutination-inhibiting antibodies in early and late immune serum from the same rabbit. Each point was calculated from the geometric mean of two titrations.

sorbed hemagglutination-inhibiting antibody to virus increased markedly on continued immunization. The ratio for neutralizing antibody likewise increased, but to a lesser extent. It appears that on continued immunization with PR8 virus there was an increase in the reactivity of both antibodies directed against the agent. The increase in reactivity of the hemagglutination-inhibiting antibody exceeded that of the neutralizing antibody. The marked increase in reactivity of hemagglutination-inhibiting antibody brought the ratios for the two antibodies much closer to equality. Sera were not available to determine whether, with more extensive immunization, the two would become equal or whether the ratio for hemagglutination-inhibition would exceed that for neutralizing antibody.

DISCUSSION

The extraordinarily steep slope of the line relating the two variables in *in ovo* neutralization of influenza virus by antibody provides confirmation of the results obtained previously by Burnet (9). It is of interest that evidence for a similarly steep slope was obtained also in *in ovo* neutralization of Newcastle disease virus (9). The implications of this relationship are important: any change in the *in ovo* virus-neutralizing titer of a serum is reflected by an enormous change, of the order of 50,000 times greater, in the *in ovo* virus-neutralizing *capacity* (7) of the serum. Under these circumstances, it is evident that serious difficulties in interpretation may arise when attempts are made to relate results obtained *in ovo* by the constant serum-varying virus technique to those obtained by the constant virus-varying serum procedure either *in vivo* or *in vitro*. In the present study all measurements of antibody concentration were carried out by the latter procedure and, as is shown, direct comparisons can be made between results obtained *in vivo* and *in vitro* under these conditions.

The finding that the correlation between hemagglutination-inhibition titers and *in ovo* neutralization titers was poor with the immune sera studied in the present investigation raised the possibility that the two procedures might not measure the same component of serum. This possibility has been considered by others (4, 5). The further finding that both the hemagglutination-inhibiting and the neutralizing titers of anti-PR8 rabbit serum, as well as convalescent human serum, were reduced in different degree by absorption with PR8 virus; that on absorption the neutralizing titer was reduced to a significantly greater extent than the hemagglutination-inhibiting titer; that the neutralizing titer could be reduced by absorption to a low level or even eliminated and yet the serum retained a high hemagglutination-inhibiting titer, provided strong evidence that the two antibodies were not identical.

Although it is obvious that hemagglutination-inhibition *in vitro* and neutralization *in ovo* are wholly different procedures, the data presented indicate that neither the poor correlation in the two titers nor the disproportionate absorption of neutralizing antibody can be attributed to differences in the precision with which the two techniques measure changes in antibody concentration. Furthermore, since discrepancies of the same order were demonstrable by neutralization in mice, it is evident that the findings are not due to peculiarities of neutralization *in ovo*.

On prolonged immunization the reactivity of both types of antibody changed. In this case, too, there was a difference between the hemagglutination-inhibiting and neutralizing antibodies for the change in reactivity of the former was much more marked than that of the latter. The changes observed in antibody reactivity on continued immunization are a further indication that the consistent, though aberrant, behavior of the two antibodies in sera obtained early in

immunization is not to be explained on the basis of differences in the techniques employed for their measurement.

As a consequence, it appears highly improbable that the antibodies measured by the hemagglutination-inhibition technique on the one hand, and by the neutralization technique on the other, are identical. If this is correct, the present findings have both practical and theoretical importance: Both the laboratory diagnosis of influenza and the evaluation of vaccines rest largely on the demonstration of an increase in the concentration of antibodies directed against influenza virus. Because hemagglutination-inhibiting and neutralizing antibodies appear in general to rise in parallel (1), use of the rapid and simple hemagglutination-inhibition technique alone may yield useful information. However, a failure to find a rise in antibody titer by the hemagglutination-inhibition technique does not necessarily indicate that there has been no increase in neutralizing antibody. Stuart-Harris and Miller (4) have pointed out some of the difficulties which may be encountered in this regard in studies of influenza epidemics.

In recent years many investigations have been carried out on the efficacy of influenza virus vaccines and particular attention has been directed to their capacity to stimulate the production of antibodies. The hemagglutination-inhibition technique has been used in the great majority of such investigations and latterly the rise and fall of antibody levels in vaccinated animals and human beings have been followed almost exclusively with this procedure. If the orientation of the antibody measurable *in vitro* is different from that measurable *in vivo*, it follows that the assessment of vaccine efficacy, on the basis of antibody levels produced, will have different significance depending upon the technique employed.

Of theoretical importance is the fact that, if two distinct antibodies directed against the virus actually develop on immunization, it can be predicted that the virus contains at least two corresponding and distinct antigens. Because it appears that influenza virus may also elaborate at least two complement-fixing antigens (14, 15), it is evident that this medium sized virus may possess an antigenic structure which approaches in complexity that of the large viruses, as for example vaccinia (16).

With the hope of separating the hypothetical antigens responsible for the production of hemagglutination-inhibiting and neutralizing antibodies, respectively, several attempts were made to bring about differential degradation of influenza virus (PR8). Although heating at 56°C. destroys the capacity of the virus to elute from RBC (17), such treatment had no significant effect on the antigenic properties of the virus. Heating at 65°C. destroys the hemagglutinating capacity of the virus (17) and also reduced its effectiveness as an absorbing antigen by about 75 per cent, but failed to cause any quantitative difference in absorptive capacity. Similarly, treatment of the virus with acid

(pH 5.0) or alkali (pH 10.0 to 11.5) was ineffective in bringing about selective inactivation of an antigenic constituent.

SUMMARY

There is an exponential linear relationship between the quantity of influenza virus neutralized and the quantity of immune serum employed in *in ovo* neutralization. The slope of the neutralization line is extremely steep. The concentration of neutralizing antibody can be measured with considerable precision *in ovo* if the constant virus-varying serum technique is utilized.

The amounts of hemagglutination-inhibiting and neutralizing antibodies which are absorbed by a given quantity of influenza virus (PR8) were found to be predictable and the degree of reactivity of these two antibodies was shown to be directly related to the extent of immunization. It was demonstrated that there are marked discrepancies in correlation between antibody titers obtained by *in vitro* hemagglutination-inhibition and *in vivo* neutralization techniques and that neutralizing antibody is preferentially absorbed by a given quantity of virus. Inasmuch as the results were found not to be attributable to peculiarities of the techniques employed, it appears that the antibodies measured by hemagglutination-inhibition *in vitro* and by neutralization *in vivo* are not identical.

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THE INHIBITION OF STREPTOCOCCAL DESOXYRIBONUCLEASE BY RABBIT AND HUMAN ANTISERA

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The fact that group A hemolytic streptococci produce an extracellular desoxyribonuclease in appreciable amounts has recently been encountered independently in two different laboratories (1, 2). The production of this enzyme appears to be a relatively constant property of these organisms, as indicated by the fact that it is elaborated by a wide variety of strains derived from different sources and with diverse biological properties. The possible significance of desoxyribonuclease in the pathogenesis of streptococcal disease is not known, although it seems certain that it does not act as a primary toxic agent.

In the case of other extracellular products of group A streptococci—such as streptokinase, streptolysin O, and hyaluronidase—it has been established that antibodies directed against them are highly effective in inhibiting their biological activity. The present investigation was undertaken to determine whether streptococcal desoxyribonuclease is similarly inhibited by its antibody and whether this antibody is commonly formed by human patients subsequent to streptococcal infections. It has previously been shown that rabbit antiserum against bovine pancreatic desoxyribonuclease effectively inhibits this enzyme (3). An opportunity was thus provided for a comparison of the antibody inhibition of two enzymes having similar action but prepared from widely different sources.

Materials and Methods

Preparation of Streptococcal Desoxyribonuclease.—The enzyme was prepared in a highly active form from supernates of cultures of a strain of group A hemolytic streptococcus. The procedure used in the preparation of the material used in most of this study is described in the following:

Fifteen liters of neopeptone dialysate broth (4) was inoculated with strain H105 and incubated at 37° for 20 hours. The cells were removed by centrifugation in a Sharples centrifuge and the slightly turbid supernate was brought to 0.4 saturation with ammonium sulfate by the addition of 243 gm. of solid salt per liter of supernate. One-tenth per cent each of filter cel and hyflo super cel were added and the suspension filtered with suction. The clear filtrate was brought to 0.8 saturation by the addition of 281 gm. of ammonium sulfate per liter of filtrate and the resulting precipitate recovered by filtration. The solution of this precipitate in 100 cc. distilled water was dark brown in color and contained practically all of the original desoxyribonuclease activity. Further fractionation was carried out with ammonium sulfate. The solution was brought to 0.4 saturation and a precipitate formed which contained most

of the colored material but only about 15 per cent of the total activity. The bulk of the activity was recovered by bringing the 0.4 saturated supernate to 0.5 saturation. The precipitate so formed was recovered by filtration, dissolved in a small volume of water, dialyzed against distilled water, and dried *in vacuo* from the frozen state. The yield was 169 mg. of dried material with an activity of 25,000 viscosity units per mg. which is approximately 2.5 times that of the amorphous desoxyribonuclease prepared from beef pancreas by the method previously described (3). It did not contain measurable amounts of streptokinase or streptococcal proteinase.

Some increase in the initial production of enzyme can be obtained by the addition of excess glucose and growth factors after overnight incubation and continued growth of the culture with neutralization of the acid formed. However, with the strain used, the increase has never been more than twofold, which is small compared to the ten- to thirtyfold increase in streptokinase concentration which occurs at the same time. The results reported by Christensen (5) using a strain of group C also indicate a disparity between the production of streptokinase and desoxyribonuclease under these conditions, although the increase in desoxyribonuclease appears to be greater than that obtained with group A strains in this laboratory.

Recovery of Streptococcal Desoxyribonuclease from Streptokinase Preparation.—As observed by Tillett, Sherry, and Christensen (1), streptokinase purified by the method of Christensen contains relatively large amounts of desoxyribonuclease. Christensen (5) has described methods for partial separation of the desoxyribonuclease from streptokinase preparations from group C strains, and analogous results have been obtained independently in this laboratory in the fractionation of group A streptokinase.

Streptokinase purified by a modification of Christensen's procedure (6) was subjected to further fractionation with ammonium sulfate. Immediately following protamine precipitation, a concentrated solution of streptokinase was brought to 0.5 saturation by the addition of an equal volume of saturated $(NH_4)_2SO_4$. The streptokinase was precipitated almost quantitatively, and subsequent precipitation of the supernate at 0.8 saturation yielded a fraction containing 60 per cent of the desoxyribonuclease and only 0.01 per cent of the streptokinase of the original material. The specific activity of the desoxyribonuclease so obtained is essentially the same as that of the preparation described above.

Preparation of Substrate.—The desoxyribonucleate used as substrate was obtained by deproteinization of calf thymus nucleohistone prepared according to the method of Mirsky and Pollister (7). Deproteinization by the chloroform method requires numerous repetitions of the process and is especially cumbersome if the preparation of moderately large amounts of material is attempted. A method has been devised to facilitate the removal of protein which is based on the procedure used for the separation of pneumococcal nucleic acid and pneumococcal polysaccharides by the use of $CaCl_2$ and ethyl alcohol (8). The amount of chloroform treatment required is greatly reduced.

The solution of nucleohistone in M NaCl is mixed with an equal volume of 0.9 M $CaCl_2$. The volume of the solution is measured and 0.2 volume of ethyl alcohol added with stirring. The desoxyribonucleate separates out as a fibrous precipitate which is removed and washed twice in a solution containing $CaCl_2$ and alcohol in the same concentration as that from which it was precipitated. The bulk of the protein remains in solution. The fibrous precipitate is redissolved in M NaCl and final deproteinization carried out by the chloroform method. Variations are experienced from lot to lot in the ease with which the last fraction of protein is removed. In some cases it is difficult to obtain nucleate which gives water-clear solutions and negative qualitative tests for protein.

Rabbit Antisera against Streptococcal Desoxyribonuclease.—The enzyme was injected intraperitoneally into rabbits in the form of an alum precipitate. Aqueous solutions of the enzyme were mixed with an equal volume of 10 per cent aluminum potassium sulfate and the solution

brought to neutrality with N NaOH to precipitate the alum. The rabbits received weekly doses of 2, 2, 10, and 10 mg. of the enzyme purified according to the method described above, and blood was obtained for serum 5 days after the last dose.

Human Sera.—The human sera were those from an epidemic of scarlet fever occurring at the Great Lakes Naval Training Center from February, to May, 1946.[1] In a previous study, the antistreptokinase and antistreptolysin O titers of these sera had been determined (9). In addition, antihyaluronidase levels of certain of these sera have been reported by Friou (10).

Measurement of Inhibition of Desoxyribonuclease.—(a) *Viscosimetric test.*—With minor modifications, the viscosimetric test is the same as that used in the measurement of the inhibition of pancreatic desoxyribonuclease by rabbit antisera (3). A stock substrate solution was prepared containing 0.1 per cent sodium desoxyribonucleate and 0.005 M $MgSO_4$ in 0.025 veronal buffer, pH 7.5. The enzyme and serum dilutions were prepared in neopeptone dialysate broth to insure stability. Although dilute solutions of the enzyme are unstable in buffer or gelatin, neopeptone broth solutions with enzyme concentrations as low as 0.02 μg./ml. show no loss of activity during incubation. Equal volumes of the appropriate enzyme and serum dilution were mixed, incubated at 37° for 30 minutes, and 0.5 ml. of the mixture was added to 4.5 ml. of the substrate solution in an Ostwald viscosimeter for measurement of residual enzyme activity.

(b) *Alcohol Precipitation Test.*—The viscosimetric method is a relatively precise method for the measurement of desoxyribonuclease activity, but it has the disadvantage of being laborious and time-consuming. It is not adaptable to the titration of the antidesoxyribonuclease activity of a large number of sera such as those obtained by serial bleedings from patients with streptococcal infections. The following method, analogous in some respects to the mucin-clot prevention test for the measurement of anti-hyaluronidase (11), was devised for this purpose. The test is dependent upon the fact that unchanged sodium desoxyribonucleate when precipitated by alcohol forms a floating fibrous mass, and that after action of the enzyme only a light flocculent precipitate is formed.

Serial twofold dilutions of the sera, beginning at 1:10, were prepared in broth, and 0.25 ml. of the dilutions was mixed in pyrex test tubes (10 × 100 mm.) with 0.25 ml. of a solution of streptococcal nuclease containing 0.2 μg./ml. After 30 minutes' incubation at 37°C., 0.5 ml. of the substrate solution, prepared as above, was added to each tube and incubation continued for 30 minutes. One ml. of ethyl alcohol was added to each tube at the end of the second incubation period and the tubes were examined for the presence of floating fibrous precipitate. The end point was defined as the highest serum dilution which prevented enzymatic degradation of the substrate so that a definite fibrous precipitate was formed upon the addition of alcohol. The final enzyme concentration in the reaction mixture (0.05 μg./ml.) is the equivalent of 5 units of enzyme in the viscosimetric system and is approximately four times the amount required to cause degradation of the desoxyribonucleate to a point where no fibrous alcohol precipitate is formed under the conditions of the test.

As in the case of other similar titrations dependent upon a twofold serial dilution system, the titers are reproducible within one tube. Consequently, in attempting to determine whether a rise in antibody has occurred, a fourfold shift in titer is considered a significant change.

Interfering Action of Serum Nuclease.—Since mammalian sera contain measurable amounts of desoxyribonuclease, it was necessary to take this fact into consideration in devising procedures in which serum was employed for its inhibitory action on streptococcal desoxyribonuclease. In the case of rabbit sera, which contain somewhat more of the enzyme than human sera, the action of the serum enzyme was detectable in the viscosimetric test at serum con-

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[1] Sera and cultures on these patients were collected during a cooperative project with the United States Naval Medical Research Unit No. 4. See reference 9.

centrations employed in the inhibition test. Heating of the diluted serum at 65° C. for 30 minutes destroys the enzyme without measurable effect on the antibody, and consequently this procedure was employed in all instances where dilutions of the serum of less than 1:50 were involved. The concentration of nuclease in human serum is too low in the vast majority of cases to be a factor at the dilution employed in this study.

<div align="center">EXPERIMENTAL</div>

Inhibition of Streptococcal Desoxyribonuclease by Rabbit Antisera.—In comparison with certain of the sera obtained from patients following streptococcal infections, the sera from rabbits injected with the purified enzyme did not possess high inhibitory activity. However, tests on sera of the two rabbits giving the best response showed that by the viscosimetric test 1.0 cc. of serum was capable of almost complete inhibition of 5 units of enzyme and better than 95 per cent inhibition of 50 units of enzyme. By the alcohol precipitation technique an end point was obtained in both cases at a serum dilution of 1:640. There is no real discrepancy between the results obtained by the two techniques, since they measure inhibition somewhat differently. By the viscosimetric technique it is feasible only to measure rather high degrees of inhibition, while by the alcohol precipitation technique a relatively small inhibitory effect would be reflected by a preservation of part of the substrate in a form that would give a fibrous precipitate.

The specificity of rabbit antibody is of some interest. Desoxyribonuclease from beef pancreas, which, in so far as has been determined, is an enzyme analogous to the streptococcal enzyme in its action on desoxyribonucleic acid and in the optimal environmental conditions required, is entirely unaffected by the presence of rabbit antiserum against streptococcal nuclease. Similarly, antibody prepared in the same manner using pancreatic desoxyribonuclease as antigen has no inhibitory action on streptococcal nuclease. These relationships are illustrated in part in Fig. 1. It will be observed that the activity of approximately 1.5 units of streptococcal desoxyribonuclease is almost completely inhibited by homologous antiserum in a final dilution of 1:25, while the same serum is without effect on a similar concentration of pancreatic desoxyribonuclease. Repeated tests using varying concentrations of enzyme and serum in an attempt to increase the sensitivity of the measurements failed to reveal even a slight degree of cross-reactivity; *i.e.*, in no case did the effect of heterologous antiserum vary significantly from the effect of normal serum.

These results have some theoretical interest since they indicate that in this case the configuration of the enzyme proteins which determines enzymatic specificity is distinct from the configuration conferring antigenic specificity. The action of the two enzymes appears to be identical, but this identity in enzyme action is not reflected by cross-reactivity in the serological studies. The same phenomenon has been observed with more favorable material by

Krebs and Najjar (12). These workers used crystalline preparations of d-glyceraldelyde-3 phosphate dehydrogenase from rabbit muscle and from yeast, and thus they were dealing with highly purified enzymes. In addition, the relative simplicity of the substrate and the enzyme reaction rendered it more certain that the enzyme action was identical in both cases. They were able
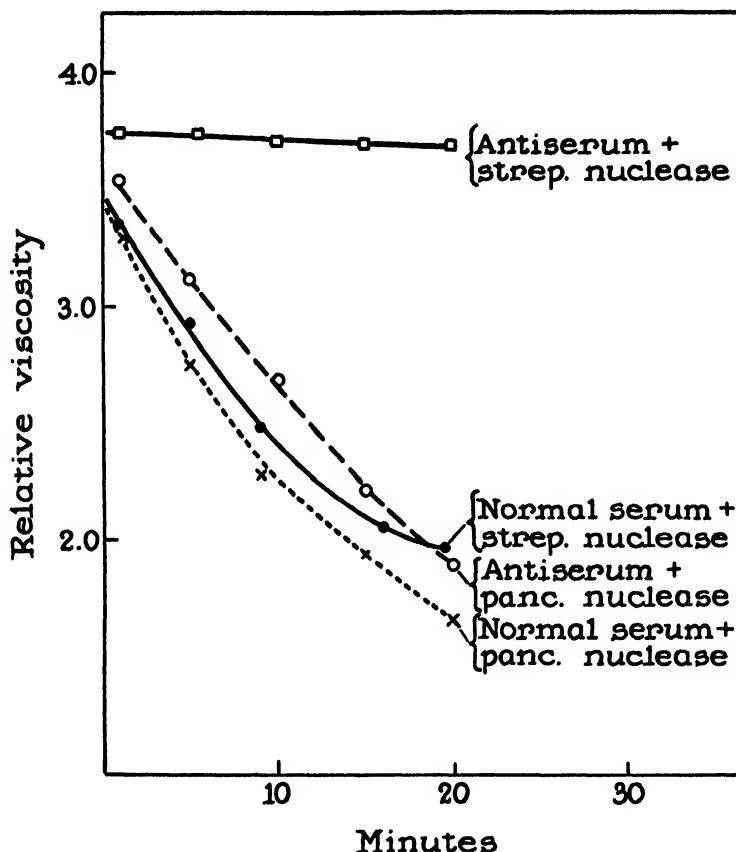
FIG. 1. Effect of rabbit antiserum to streptococcal desoxyribonuclease on the activity of streptococcal and pancreatic desoxyribonucleases.

to show that rabbit antiserum against yeast enzyme caused a marked degree of inhibition of the homologous enzyme but did not affect the activity of the rabbit muscle enzyme.

The specificity of rabbit antibody against streptococcal desoxyribonuclease has been further demonstrated with respect to its action on other extracellular products of hemolytic streptococci. For example, the activity of streptokinase and streptococcal hyaluronidase is not affected by the antiserum.

Inhibition by Human Sera.—In approaching the question of whether the human subject produces antibody to streptococcal desoxyribonuclease following streptococcal infections, initial studies were made employing the viscosimetric test. It was found that although a high degree of variability existed in the apparent antibody response of various individuals following scarlet fever, the development of antibody against this enzyme was pronounced in some cases.
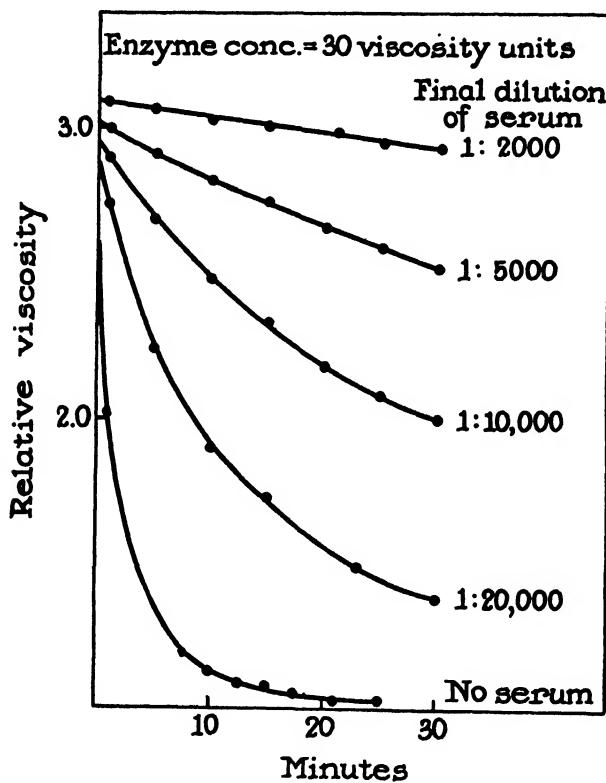
Fig. 2. Effect of human serum (obtained from patient 4 weeks after onset of scarlet fever) on streptococcal desoxyribonuclease. Constant enzyme concentration tested against varying dilutions of serum.

An example of one of the latter cases is illustrated in Fig. 2. Serum obtained from this patient at the onset of scarlet fever was totally devoid of the capacity to inhibit the enzyme, and subsequent bleedings taken at weekly intervals showed a progressive increase in this property. The serum used in the experiment illustrated in Fig. 2 was obtained 4 weeks after onset of the infection and represents the peak of antibody response. The inhibitory effect of the serum is further emphasized by the fact that a relatively high concentration of enzyme

for the viscosimetric test—*i.e.*, 0.25 μg./ml. or 30 units—was employed. Fig. 2 shows that even at a final serum dilution of 1:20,000, the serum causes a highly significant inhibition of the enzyme (90 per cent) and that with increasing concentration of serum up to 1:2000 complete inhibition (99.6 per cent) is approached.

The specificity of human antibody to streptococcal desoxyribonuclease is comparable to that of the rabbit antisera. Sera with high concentration of antibody, such as the one illustrated in Fig. 2, have no inhibitory action on pancreatic desoxyribonuclease or on a sample of crude desoxyribonuclease prepared from Pneumococcus (Type IIR).

The frequency with which patients respond by formation of antibody to desoxyribonuclease and the relative magnitudes of the responses were determined by applying the alcohol precipitation test to the sera of 90 patients from an epidemic of scarlet fever at the Great Lakes Naval Training Center. The antibody response of these same patients to streptokinase and streptolysin O had previously been determined, making possible a comparison of the antidesoxyribonuclease data with those of two more commonly studied streptococcal antibodies (9).

The results of the antinuclease determination show certain interesting differences from those obtained in the case of the other antibodies.

1. The percentage of patients showing a significant response to desoxyribonuclease (38 per cent) was definitely lower than in the case of streptokinase (61 per cent) and streptolysin O (70 per cent). Thirty-four of the 90 patients (33 per cent) did not have measurable amounts of antibody to desoxyribonuclease by this relatively sensitive test at any time during the period in which sera were obtained. It would appear, therefore, that human beings respond by antibody formation to this extracellular antigen less frequently than to certain others, despite the fact that desoxyribonuclease production is a rather constant property of hemolytic streptococci. Strains isolated from patients in this epidemic were among those tested for production of the enzyme *in vitro*, and all these elaborated the enzyme in an amount comparable to that of the various stock laboratory strains.

2. The values obtained for antidesoxyribonuclease titers are scattered over a wider range than in the case of the other two antibodies. Fifty-three of the 90 patients had no demonstrable antinuclease at the time of onset of the scarlet fever and while many of these patients showed no subsequent rise others had increases in antibody up to as high as a serum dilution of 1:10,240. In the study of the antistreptokinase and antistreptolysin O titers it was shown that the mean titers of those patients who developed rheumatic fever subsequent to scarlet fever were significantly higher than those of the patients with uncomplicated scarlet fever. The spread of the antidesoxyribonuclease titers renders a comparable use of mean titers valueless in this small series of cases,

and it can only be said that analysis of the data suggests that a comparable relationship holds in this case; *i.e.*, that the rheumatic subjects *on the average* have a greater antibody response to desoxyribonuclease.

3. The large number of patients showing no demonstrable antinuclease at the time of onset of scarlet fever is at variance with the fact that the great majority of the patients had appreciable antistreptokinase and antistreptolysin

TABLE I

Antidesoxyribonuclease Titers of Twenty-Three Patients with Scarlet Fever Followed by Rheumatic Fever

Case No.	Antidesoxyribonuclease titer				
	Time after onset of scarlet fever, *wks.*				
	0	1	2	3	4–6
13	0	40	1280	10,240	10,240
16	320				640
23	0		0	0	0
30	10			10	20
51	80			80	80
52	0			20	320
65	0			40	0
88	10			80	80
92	20			40	20
120	0			80	640
234	160			320	160
247	80			160	160
251	0			10,240	10,240
61	20			1280	5120
42	0			0	0
45	0			0	0
181	40			80	80
175	40			40	20
164	0			0	0
173	0			640	640
217	0			0	0
337	40			320	40
44	0			0	0

O titers at this time, presumably as the result of previous experience with the hemolytic streptococcus. An explanation which suggests itself for this finding is that antidesoxyribonuclease may be less stable and thus disappear more rapidly from the body than the other two antibodies. However, no support for this explanation could be obtained from antibody studies in a few instances in which sera were available for many months following the scarlet fever. The rate of decrease of antidesoxyribonuclease titer in these cases was comparable to that of antistreptokinase.

Certain of the facts summarized above are illustrated in Table I, in which

the titers of antidesoxyribonuclease (as reciprocals of the serum dilution) are given for one group of the 90 patients. This group (designated group V in
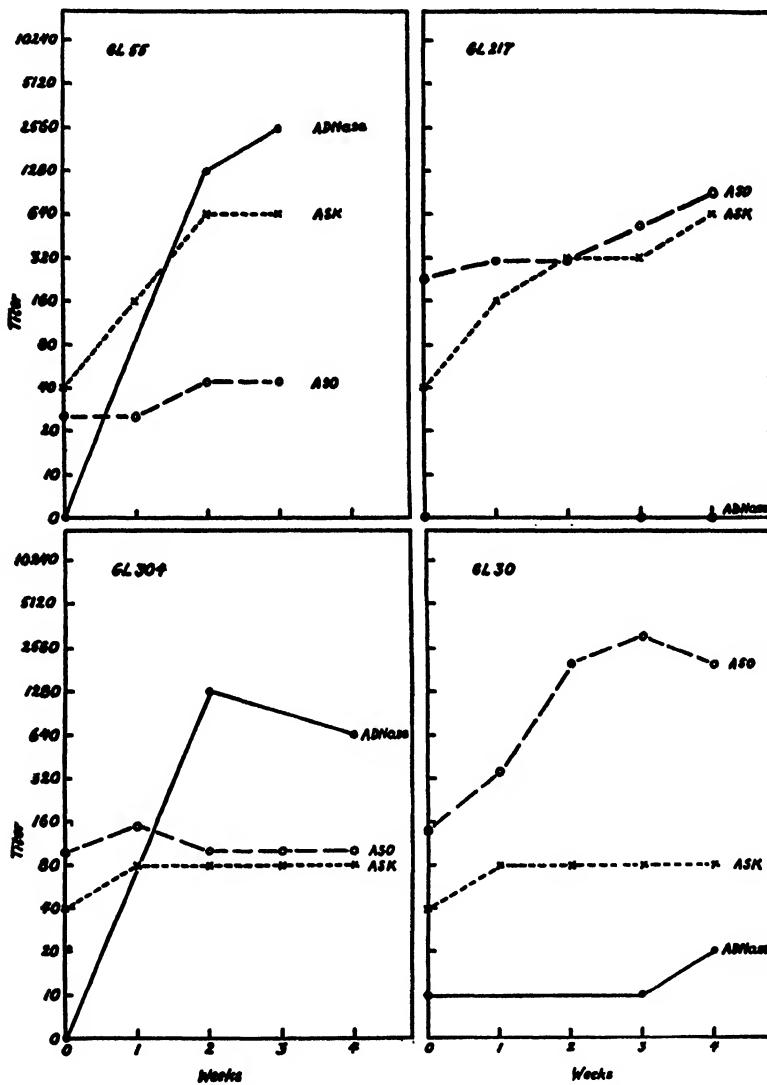
FIG. 3. Comparison of antidesoxyribonuclease response with antistreptokinase and antistreptolysin 0 titers in four patients following scarlet fever. ASK= antistreptokinase, ASO-antistreptolysin 0, and ADNase= antidesoxyribonuclease.

reference 9) represents those patients who developed rheumatic fever as a sequela of the scarlet fever. The case numbers are given so that direct comparison can be made with the data recorded in reference 9.

Observations previously made concerning the suppressive effect of penicillin therapy on antibody response to extracellular antigens of the streptococcus are confirmed by the antidesoxyribonuclease data. In a group of 17 patients who received "successful" penicillin therapy as indicated by the prompt and permanent disappearance of hemlytic streptococci from the nasopharynx, none had significant rises in antistreptokinase and there were no rises as great as fourfold in the antistreptolysin O titer. Similarly only one of the 17 patients showed a significant rise in antidesoxyribonuclease titer.

Independent Variation of Antibody Response to Different Antigens.—Inspection of the antibody data from the 90 scarlet fever patients indicates that practically all possible combinations of antibody response to the three extracellular antigens occurred. This fact is illustrated by the results in four of these cases which are charted in Fig. 3. It will be noted that in case 55 excellent responses occurred to streptokinase and desoxyribonuclease, while there was no significant increase in antistreptolysin O. In case 217 there was no response to nuclease but a good response to the other two antigens, and conversely in case 304 there was a response only to nuclease. An example of a patient showing a significant response only to streptolysin O is provided by case 30. In so far as *in vitro* tests can be relied on, it has been shown in the case of at least two of these antigens—streptokinase and desoxyribonuclease—that the variations are not attributable to quantitative differences in the amount of antigen produced by the strains. No study was made of the relative *in vitro* production of streptolysin O. The most reasonable assumption is that the variations represent individual differences in the ability of the host to respond by the formation of antibody to a given streptococcal protein. From the theoretical point of view it is of interest that the quantitative differences in antibody response do not merely reflect a general characteristic of the host that applies to all antigens of the streptococcal cell, but they are specifically determined also by the nature of the individual antigen.

SUMMARY

Rabbit antisera against partially purified streptococcal desoxyribonuclease inhibit the action of the enzyme on its substrate. The activity of pancreatic desoxyribonuclease is not affected by these antisera. Similarly antibody against pancreatic nuclease does not inhibit the streptococcal enzyme.

Certain patients develop inhibitory antibody to streptococcal desoxyribonuclease following streptococcal infections, occasionally in very high titer, although the proportion of patients showing an antibody response appears to be lower than in the case of streptokinase and streptolysin O.

The pattern of antibody response to desoxyribonuclease has been compared to that of streptokinase and streptolysin O in a group of ninety patients from an epidemic of scarlet fever.

The author takes pleasure in acknowledging the able technical assistance of Miss Elizabeth Van Pelt.

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THE SIGNIFICANCE OF THE UREA CLEARANCE

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By the courtesy of Mr. H. Fairfield Smith we have had the privilege of examining in advance of publication his paper "Urea Clearance Tests." This paper appears to touch on three separate questions: (1) What does the clearance purport to measure? (2) What factors affect the clearance? (3) What is the diagnostic and prognostic significance of the clearance in renal disease? Inasmuch as the available data appear to have been only partially available to Mr. Smith, the writers venture to add the following comments on these questions.

What Does Urea Clearance Measure?

The urea clearance measures the efficiency with which the kidneys remove urea from the blood stream, when due correction is made for the effects of low rates of urine flow. Studies on man and animals have shown that the urea clearance measures the rate of filtration of urea in the glomeruli minus the rate of urea reabsorption by the tubules (Van Slyke et al., 1934, 1935; Smith, 1937; Chasis and Smith, 1938; Shannon, 1936).

Factors Affecting Clearance

Like all physiological functions, the urea clearance varies under different physiological conditions, particularly those which affect the renal blood flow (Van Slyke et al., 1934, 1935) and the urine volume (Van Slyke, 1947; Möller et al., 1928; Dole, 1943). Consequently the clearance of a given subject may vary by as much as ±20% of his average under the usual conditions of daily life (Möller et al., 1928). The variation of the clearance with urine flow has been studied (Van Slyke, 1947; Möller et al., 1928; Dole, 1943) and shown to be due to variations in tubular reabsorption of urea that are predictable by the simple diffusion laws (Dole, 1943). High protein diets have been shown to increase renal blood flow and urea clearance in dogs (Jolliffe et al., 1931), and partial protein starvation has been shown (Cope, 1933) to decrease the clearance in men, a finding which is confirmed by Mr. Smith's observations.

Variability is shown by other physiological functions, measurement of which is nevertheless of value in assessing clinical conditions: e.g., the metabolic rate, body temperature, and pulse rate vary with physical activity, excitement etc. Such variability does not invalidate the measurement of these functions, or of the urea clearance, in assisting to estimate the condition of a patient.

Renal disease affects as a rule chiefly the rate of filtration, which decreases as glomerular destruction advances, although in advanced nephritis increased permeability of the tubules appears to add its effect by increasing urea reabsorption, particularly when urine volume is low (Van Slyke, 1947). Damage to the tubules by nephrotoxic substances (Richards, 1929; Lucké, 1946), or by renal ischaemia, such as occurs in severe and prolonged shock (Phillips et al., 1946; Van Slyke, 1948; Phillips et al., 1948) appears to make the tubules more permeable to back diffusion of urea into the blood, and lowers the clearance by increasing urea reabsorption. In all these conditions the urea clearance has been found of value in assisting the appraisal of the extent of renal damage, and the course of progress either towards recovery or fatal renal failure.

The Diagnostic Significance of Urea Clearance in Renal Disease

Neither the urea clearance, nor any other physiological measurement, should be asked to serve as the sole criterion to discriminate between health and disease. The clinician using such a test must evaluate the results in terms of all known causes of variation, physiological and pathological. As the increase of metabolic rate with muscular or digestive activity or excitement does not invalidate its use in the thyroid clinic, so the decrease of the urea clearance with protein starvation or shock (Phillips et al., 1946; Van Slyke, 1948) does not invalidate its utility in following the course of renal damage or disease, when proper allowance is made for the effect of extra-renal influences.

Studies of a considerable number of cases of renal disease of different types, some of which were observed over periods of years, have led (Van Slyke et al., 1930) to the following conclusions:

"In acute hemorrhagic nephritis fall of the urea clearance to as low as 10% of normal was found not inconsistent with apparently complete recovery. . . .In all these cases which recovered or improved, however, the blood urea clearance began to rise within four months after the acute onset. . . .Of the different features of (advanced renal) disease that were followed, the blood urea clearance proved to be the most closely related to the onset of final renal failure. The renal function, measured by the clearance, could apparently remain indefinitely at 10% of normal without uremia; but when it fell to 5% uremia occurred, and was usually fatal. Exceptions to the immediately fatal outcome were found in acute cases, which can recover if the functional depression does not last too long, and occasionally a terminal case, in which functional fall was partly due to factors, such as desiccation, other than destruction of renal tissue. In such a case treatment, particularly saline and glucose injections, may both improve the general condition and somewhat increase the urea clearance, although the added lease of life appears to be at most a few months."

These observations have been confirmed and extended in subsequent studies continued to the present time in this clinic, and will be presented in a future publication.

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THE EFFECT OF RIGID SODIUM RESTRICTION IN PATIENTS WITH CIRRHOSIS OF THE LIVER AND ASCITES

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Low salt diets have been used for many years in the treatment of edema and ascites in patients with cirrhosis of the liver. Enthusiastic reports appeared in the French literature as early as 1904 claiming actual termination of ascites formation from the use of a low salt diet.[1, 2, 3] In recent years, however, emphasis on high protein, high caloric diets in treatment of cirrhosis has led many physicians away from the use of low salt regimens, since it has seemed difficult to devise a palatable low salt diet which is adequate in protein. Moderate restriction of salt has continued to be common practice in the treatment of ascites but results have not been striking.

Recently, numerous reports have appeared dealing with the role of sodium in the formation of ascites and edema in cardiac disease.[4, 5, 6] Sodium and water retention by the kidney has been claimed as a factor in the production of ascites in cirrhosis as well as in congestive heart failure by Farnsworth[7]. Layne and Schemm[5, 8] have applied this principle to the management of cardiac and cirrhotic ascites by means of low salt diets. Chalmers and Davidson[9] have shown in a preliminary study of sodium balance in cirrhosis that salt plays an important role in fluid retention. Recently, the work of Whipple and co-workers[10] on dogs rendered ascitic by constriction of the inferior vena cava above the liver has demonstrated that ascites of purely mechanical origin can be affected markedly by variations in salt intake.

The role of hypoalbuminemia in the formation of ascites in patients with liver disease has been recognized by numerous observers.[11, 12] Ralli and co-workers[13] have obtained evidence that increased excretion of antidiuretic substance also plays a role in ascites formation. Recently, the work of Blakemore[14] has demonstrated the importance of portal hypertension as a third factor in the formation of ascites. The present report represents a description of the direct relationship between the sodium content of the diet and the accumulation of ascites in thirteen patients despite the operation of the other three factors. Detailed studies of electrolyte balance in three of these patients will be published in a separate communication.

Materials and Methods

The thirteen patients to be discussed were admitted to the Hospital of The Rockefeller Institute at least ten days prior to the initiation of salt restriction in the diet.

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The diagnosis of cirrhosis of the liver was established by characteristic history, numerous physical examinations, x-ray studies of the esophagus and abdomen, and a wide variety of liver function tests. Ascites had been present for four to forty-eight months prior to the onset of low salt therapy and had necessitated two to seventy-eight paracenteses. Fluid continued to accumulate at the usual rate at the time of onset of therapy.
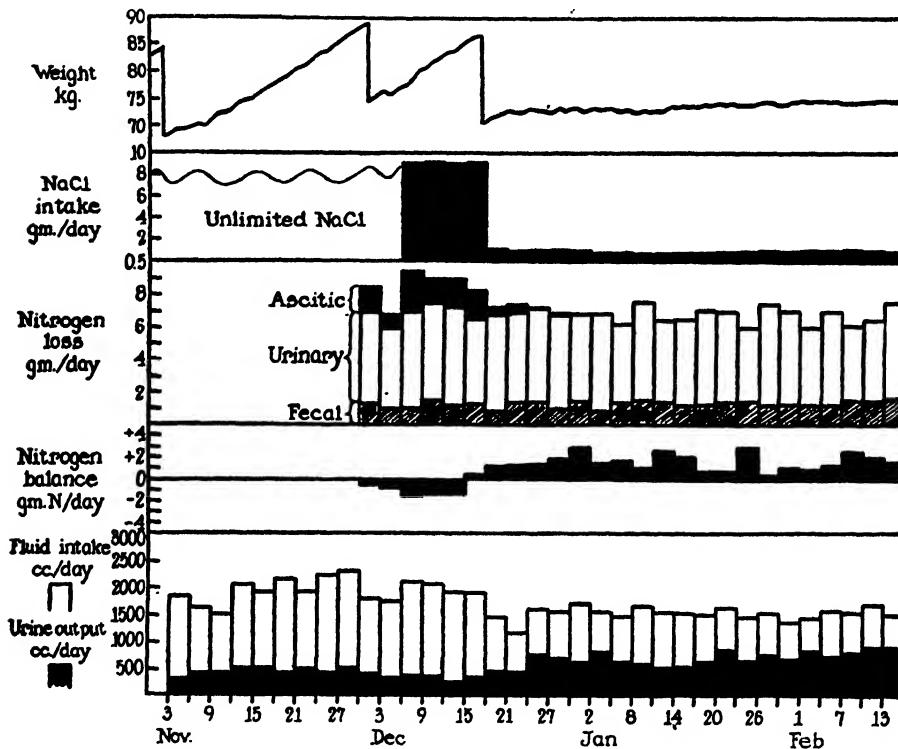
FIG. 1.—Case 1. Three paracenteses in the hospital prior to Na restriction. Immediate cessation of ascites formation on diet of 1 Gm. of NaCl per day. Reversion from negative to positive nitrogen balance.

The etiology of the cirrhosis was chronic alcoholism with associated nutritional disturbance in nine of the thirteen patients. None of the patients was suffering from an enlarged, acutely decompensated fatty liver at the time of admission.

The diets used were prepared by trained dietitians who kept careful records of the daily intake of protein, fat, carbohydrate, and salt. An effort was made to provide patients with an excess of food and calculations were made from the amounts offered and refused. Protein, salt, and caloric intake were calculated from standard tables. The sodium intake was checked by direct analyses. In the detailed studies on sodium balance, direct analysis of the sodium content of the food, urine, and feces was carried out. (NaCl was calculated on the basis of measurements of Na.) In the low salt diets,

special attempts were made to keep the food palatable. Salt-free bread, available for purchase, was used. No salt substitutes were administered. Where protein intake tended to be low, or where a high protein, low sodium intake was sought, whole protein supplements were added to the diet.* As much as 50 Gm. of protein per day could be provided with these palatable supplements. Serum albumin was determined by a modification of the specific immunologic method of Chow.[15] Instead of measuring turbidity, the antigen-antibody precipitate was determined by means of the ninhydrin reagent of Moore and Stein.[16] The Howe method with Kjeldahl digestion was also used for protein determination. Osmotic pressures were determined by a specially constructed Hepp type osmometer. Determinations of serum bilirubin, bromsulfalein retention, thymol turbidity, and total lipids were also carried out in all of the patients during the period of therapy. The methods employed were identical with those presented in a previous study.[17] Plasma chlorides were determined by the method of Van Slyke[18]; plasma volume, by the method of Gibson and Evans.[19] Sodium was determined by means of a Perkin-Elmer flame photometer utilizing lithium as an internal standard.

RESULTS

CASE 1.—A 52-year-old man with cirrhosis of unknown etiology beginning six months prior to admission and requiring six paracenteses. Physical examination showed occasional spider angiomata and a small, hard liver. Laboratory tests indicated an advanced cirrhosis. The total protein was 5.7 Gm. per cent, with albumin 1.9 Gm. per cent. Bromsulfalein retention was 37 per cent in 45 minutes. Serum esterase was 22 units. Cephalin flocculation was 2+; the thymol turbidity was 10 units and bilirubin was 1.8 mg. per cent.

On admission the patient continued to accumulate ascites at a regular rate (Fig. 1), necessitating three paracenteses while on an unlimited NaCl intake. This was found by analysis to average 9.2 Gm. NaCl per day. Urinary excretion of NaCl was extremely low, averaging 0.04 Gm. per day, while fecal NaCl was 0.3 Gm. per day. The patient was in negative nitrogen balance on the normal diet. This was chiefly due to the loss of ascitic nitrogen through the paracenteses.

Immediately following the initiation of a low salt diet which on analysis was found to contain between 0.8 and 1.2 Gm. NaCl per day, the ascites formation ceased and the patient reverted to positive nitrogen balance as indicated in Fig. 1. The urinary output approximately doubled and both the urinary and fecal NaCl concentrations fell. The serum and ascitic fluid Na levels showed a fall of approximately 3 meq. per liter. After the patient remained on this diet for three months, the urinary NaCl which had remained constant suddenly increased and the patient lost considerable ascites. Table I illustrates the sudden rise in the urinary NaCl despite continued NaCl restriction. The dietary intake improved progressively during this time and positive nitrogen balance increased. At the present time, six months after the initiation of Na restriction, the patient is markedly improved although not entirely recovered. He now tolerates 4 and 5 Gm. NaCl per day without forming ascites because of the increased urinary output of Na.

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* Delcos Granules (Sharp & Dohme); Melactin (Squibb); Protinal (National Drug).

The total protein in the serum and ascitic fluid rose by 2 Gm. per cent after five months on the diet.

Comment.—Rigid NaCl restriction caused a cessation of ascites formation, positive nitrogen balance, and a rise in serum and ascitic fluid proteins. Urinary NaCl increased with the mobilization of ascitic fluid. A five-month period of therapy was necessary before the patient showed signs of recovery.

CASE 2.—Fig. 2 illustrates the course of a 42-year-old woman with a nutritional cirrhosis who had ascites for eight months prior to admission. She had a total of twenty-nine paracenteses in that period, during which time she was at home on minimal activity. Since an accurate record of these was kept, they are indicated in the weight curve of Fig. 2. Her diet, poor at the onset of the ascites, was adequate for six months prior to admission. The skin showed numerous spider angiomata and prominent liver palms. The liver edge was barely palpable at the costal margin. An esophagram showed esophageal varices. Laboratory tests likewise pointed to an advanced cirrhosis. Pro-

TABLE I

	NaCl Gm./Day			Na MEQ./Liter	
Date	Food	Urine	Feces	Serum	Ascitic Fluid
Dec. 3............	9.1	0.05	0.30	138	137
Dec. 15......	9.3	0.07	0.35	139	139
Jan. 12.....	1.1	0.02	0.15	135	135
Feb. 12...... .. .	0.9	0.02	0.12	135	134
Mar. 24............	1.0	2.00	0.25	137	137
May 12............	0.9	1.00	0.18	136.	135

teins formed by the liver were all low; plasma albumin was 2.4 Gm. per cent, fibrinogen 225 mg. per cent, prothrombin 38 per cent of normal, and esterase 13 units. Gamma globulin was elevated as indicated by a zinc turbidity of 32 units and a total serum globulin of 3.5 Gm. per cent. Bromsulfalein retention was 40 per cent in 45 minutes. Ascitic fluid total protein was 1.0 Gm. per cent, with albumin 0.7 Gm. per cent.

On admission the patient was placed on a regular hospital diet and activity equivalent to that at home. On this regimen she continued to gain weight and ascites at her usual rate. The urinary output was small. Following a paracentesis which became necessary, she was put on a diet with NaCl restricted to 1 Gm. per day. The response was immediate with a complete cessation of ascites accumulation and a rise in urinary output to about three times the previous output. This occurred despite a spontaneous decrease in fluid intake. A small amount of ascites, detectable by shifting dullness, persisted. This effect was sustained throughout her hospitalization and at home. After three months the patient was able to tolerate larger amounts of NaCl in her diet without forming ascites. She has remained well during the year since discharge and all ascites has disappeared.

A rise in serum albumin from a subnormal level of 2.4 Gm. per cent to normal levels of 4 to 4.5 Gm. per cent occurred.

Clinically there was marked improvement in this patient. As soon as she became adjusted to the diet, she complained only of the flat taste of food without salt but this did not interfere with her eating a perfectly adequate diet. The termination of her need
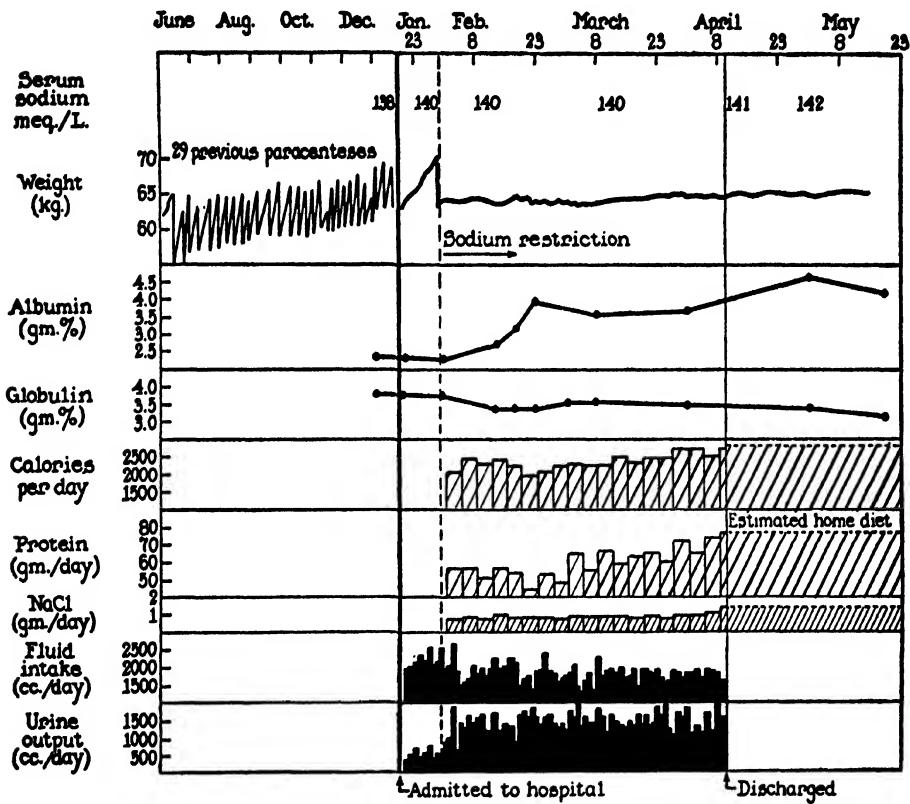
Fig. 2.—Case 2. Ascites of eight months' duration requiring thirty paracenteses. Immediate control of ascites with salt restriction. Prompt increase in urinary output and delayed rise in serum albumin.

for paracenteses after having had thirty was a strong factor in raising her morale and insuring cooperation.

Comment.—A low salt diet aided in restoring this patient to a fairly normal, active life, after having previously required almost weekly paracenteses for seven months. The urinary output likewise showed an immediate increase, while the serum albumin levels gradually rose to almost double the former levels within three weeks.

CASE 3.—Fig. 3 illustrates the course of a 54-year-old wine drinker who on admission to the hospital was accumulating ascitic fluid at a regular rate. He had been hospitalized elsewhere on two occasions. Following an unexplained febrile episode he lapsed into hepatic coma for four days but gradually recovered. Three paracenteses were performed at the other hospital following the episode of coma. Since weights were available, these are illustrated in the weight curve of Fig. 3. On physical examination the patient showed a small, hard liver and marked ascites with no edema. Following admission to the Rockefeller Hospital he was kept on a regular diet with normal sodium intake for three weeks. Ascitic fluid continued to accumulate and a fourth para-
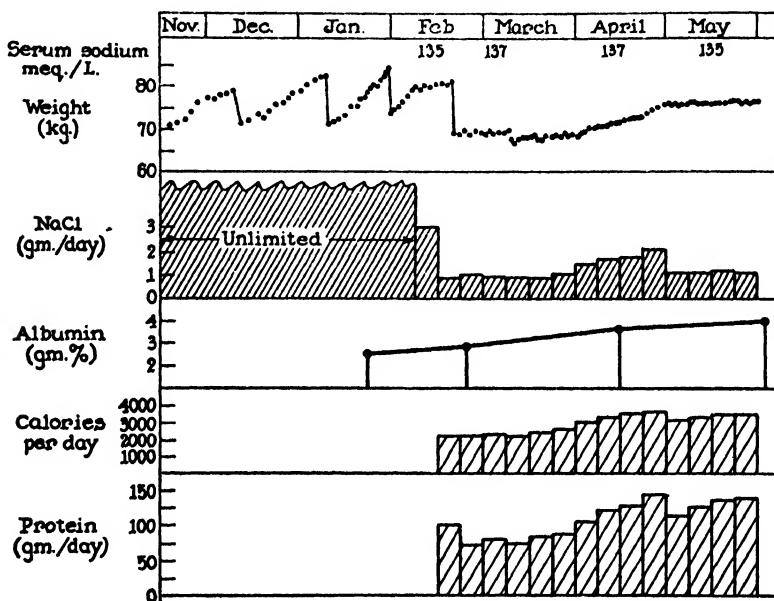
FIG. 3.—Case 3. Ascites formation controlled by NaCl restriction at 1 Gm. per day. Reaccumulation of ascites on 1.5 to 2 Gm. NaCl per day. Control of ascites again at 1 Gm. per day. High protein intake (140 Gm. per day) despite salt restriction to 1 Gm. per day.

centesis was performed. Following the reduction of the NaCl content of his diet to 3 Gm. per day, there was an immediate slowing in the rate of ascites formation as is readily visible from the weight curve of Fig. 3. The NaCl content was further reduced to approximately 1 Gm. per day, and following a fifth paracentesis the patient ceased accumulating ascitic fluid for one month. The NaCl content of the diet was then gradually raised to 2 Gm. per day. Ascitic fluid began to accumulate and the weight curve illustrated in Fig. 3 can be seen to rise with progressive increments of salt intake. When NaCl was reduced again to 1 Gm. per day, ascitic fluid again ceased accumulating and the weight curve flattened out. By means of protein supplements, as much as 140 Gm. of protein were supplied a day during this latter period despite the very low NaCl intake.

Improvement on the low salt regimen was very striking. The discontinuance of paracenteses caused the patient to feel better and to eat better and resulted in a rise in the albumin concentration in the serum. The limitation of salt was very well tolerated. After three months on the low sodium diet, the patient recovered sufficiently to tolerate a normal diet at home without ascites.

Comment.—The degree of salt restriction necessary to control the ascites in this patient was well defined at about 1.3 Gm. a day. Limitation below this level promptly resulted in a favorable therapeutic response with control of ascites and a marked increase in urine volume. By the use of protein and carbohydrate supplements it was possible to raise the patient's protein intake to 140 Gm. per day and his caloric intake to over 3,000 calories despite limitation of sodium chloride to 1 Gm. per day.

CASE 4.—Fig. 4 illustrates the course of a 60-year-old housewife who developed hepatic cirrhosis without known cause and had been steadily accumulating ascites for four years. She had had seventy-six paracenteses prior to admission, with removal of an average of 10 liters per tap followed by leakage of fluid from the wound for one to two weeks. After closure of the wound she consistently accumulated approximately 1 liter of ascitic fluid per day. Edema of the legs appeared as the intra-abdominal pressure increased.

In the nine months prior to admission the patient had become progressively weaker. She had lost considerable body tissue and had had several episodes suggesting minor cerebrovascular accidents. In the course of the four years the patient had been hospitalized for all paracenteses, and the main therapeutic efforts were directed toward a highly nutritive diet with occasional trials of mercurial diuretics.

On admission she appeared chronically ill, with marked tissue wasting. The liver was slightly enlarged and hard. Laboratory findings were consistent with an advanced cirrhosis with a decrease in the proteins formed by the liver; serum albumin was 2.5 Gm. per cent, prothrombin level 47 per cent of normal, fibrinogen 160 mg. per cent, serum esterase 27 units. Bromsulfalein retention was 23 per cent. Ascitic fluid total protein was 1.1 Gm. per cent, with albumin 0.5 Gm. per cent.

On the hospital diet with unrestricted salt the patient continued to gain weight and ascites at her usual rate until two paracenteses were performed. Just before the second tap in the hospital, salt in the diet was limited to 1 Gm. a day. The rate of accumulation of ascites was promptly slowed and eventually leveled off at a point just short of requiring a paracentesis. With the institution of the low salt diet, urine output which had been minimal (200 to 300 c.c. a day) except following mercurials approximately doubled. This occurred despite a voluntary reduction in fluid intake. Attempts to raise the fluid intake were futile, leading to nausea. This may have been due to impaired renal function presumably on an arteriosclerotic basis. Both prior to and during the period of sodium restriction the patient showed a constantly elevated nonprotein nitrogen, a urea clearance of 25 per cent, and urine specific gravity fixed at 1.010. The patient's diet averaged 60 Gm. of protein and 2,000 calories daily, with NaCl limited to below 1 Gm. a day.

Laboratory tests showed no change with the exception of a moderate rise in serum albumin levels, from 2.5 Gm. per cent to 3.7 Gm. per cent. During the entire course

there were but insignificant variations in the hemoglobin levels. Clinically this patient improved for two months but then her dietary intake fell and she was returned to a normal diet. A second period on the low Na diet again proved of little permanent value and the patient died three months after returning to the normal Na intake.

Comment.—The chronicity of this patient's illness with resultant debility and prolonged ascites plus the renal and cerebral arteriosclerotic complications provided a difficult test for this regimen. After seventy-eight paracenteses the
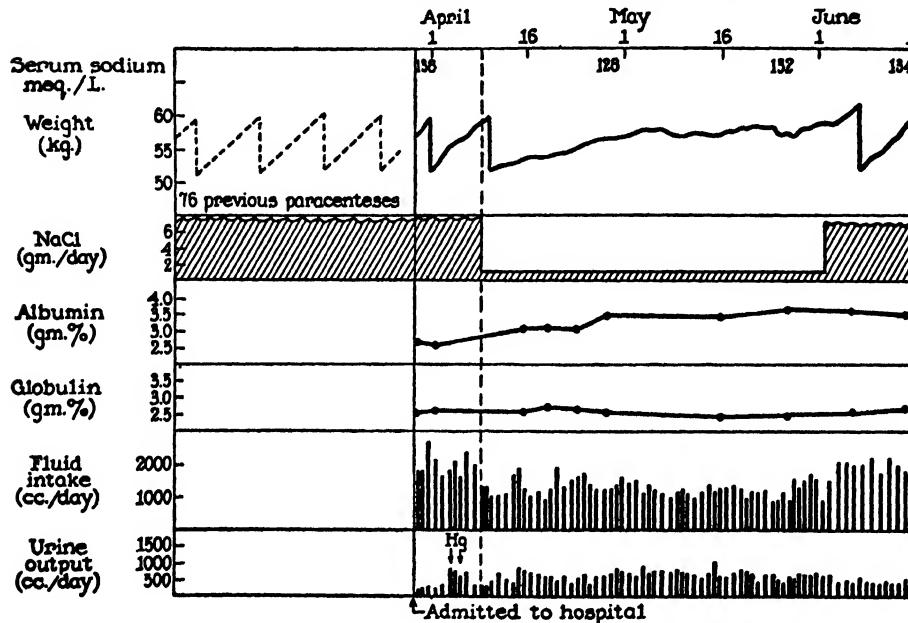
Fig. 4.—Case 4. Ascites of four years' duration requiring seventy-eight paracenteses. Effect of salt restriction in controlling formation of ascites. Increase in urine output and serum albumin level. Return of ascites and need for paracenteses on normal diet.

rate of ascites formation was promptly slowed by salt restriction, but the dietary intake was poor and the patient eventually died.

DISCUSSION

The results of rigid Na restriction have been described in detail in four of the thirteen patients studied. The remainder of the patients showed similar results with complete cessation of ascites formation when the NaCl was limited to 1 Gm. per day. Higher intakes caused ascites formation in direct proportion to the NaCl given. An average critical level of NaCl intake was obtained for these patients above which ascites formation occurred. This was 1.2 Gm. NaCl per day. The reason for this direct relation between NaCl intake and ascites was

that the NaCl excretion in the urine was extremely low regardless of how much NaCl was furnished in the diet. The urinary NaCl excretion in eight of these patients on a diet of 7 to 9 Gm. NaCl per day ranged from 0.02 to 0.13 grams. Fecal NaCl excretion was higher, averaging 0.35 Gm. per day. With this low excretion of NaCl it is apparent that the large part of the NaCl intake is used to form ascitic fluid. Insensible NaCl loss was not measured but could be calculated from the NaCl balance data to be approximately 0.8 Gm. per day.

Excretion of water rose in all of the patients on the low sodium diet. When there was no Na supplied in the diet for ascitic fluid accumulation, water was excreted proportional to the intake. A high fluid intake had little effect in altering daily sodium excretion.

All but one of the thirteen patients were able to tolerate the low Na regimen for at least three months without ill effects. The one exception, Patient 4, showed a fall in fluid and caloric intake on the diet. Another patient developed muscle cramps after being on the diet for five months. These were relieved by supplying Na. The patient had done well on the diet without forming ascites prior to the onset of the cramps. Serum Na levels were followed in all of the patients. A fall in serum Na of approximately 3 meq. per liter usually occurred. One patient who received mercurials in addition to the low Na diet showed the greatest fall, 12 meq. per liter.

Nitrogen balance studies were carried out in two of the patients. These patients were in negative balance prior to Na restriction because of the loss of ascitic nitrogen. When ascites formation ceased on the low Na diet, the patients reverted to positive nitrogen balance.

All of the patients demonstrated a rise in serum proteins on the low Na diet. Fig. 5 shows a group of determinations on the serum and ascitic fluid of one of the patients (Case 1). The rise in albumin and in colloid osmotic pressure was approximately equal in the two fluid compartments, demonstrating the equilibrium which exists. The total protein of serum and ascitic fluid rose considerably more than the albumin. Plasma volume determinations showed little change and thus did not account for the rise. Some of the other patients demonstrated a 10 per cent fall in plasma volume accounting for a portion of the protein rise. The patients who responded best to the diet and gained body weight soon after treatment was started showed a rise in serum albumin that was sometimes greater than the total protein rise. This was due to a fall in globulins. The selective rise in albumin was a sign of improvement in liver protein synthesis. This did not occur in the chart shown (Fig. 5) nor in most of the other patients. The rise in these patients of all the protein components appeared to be the result of cessation of protein loss in the ascitic fluid permitting the proteins to accumulate in the body.

The use of newly available low Na protein supplements permitted the administration of as much as 150 Gm. of protein per day on the low Na diet.

The patients showed considerable individual variation in their ability to take different supplements, but of those which were used there usually was one which was readily tolerated by each patient. Improvement appeared to be more rapid when the protein was kept above 100 grams. However, some of the patients responded well at a level of 70 Gm. of protein per day.

The general clinical results of a three-month period on the low NaCl diet were evaluated by studying the effect of normal Na intake on ascites at the end of the three months. Four patients recovered sufficiently during this time to
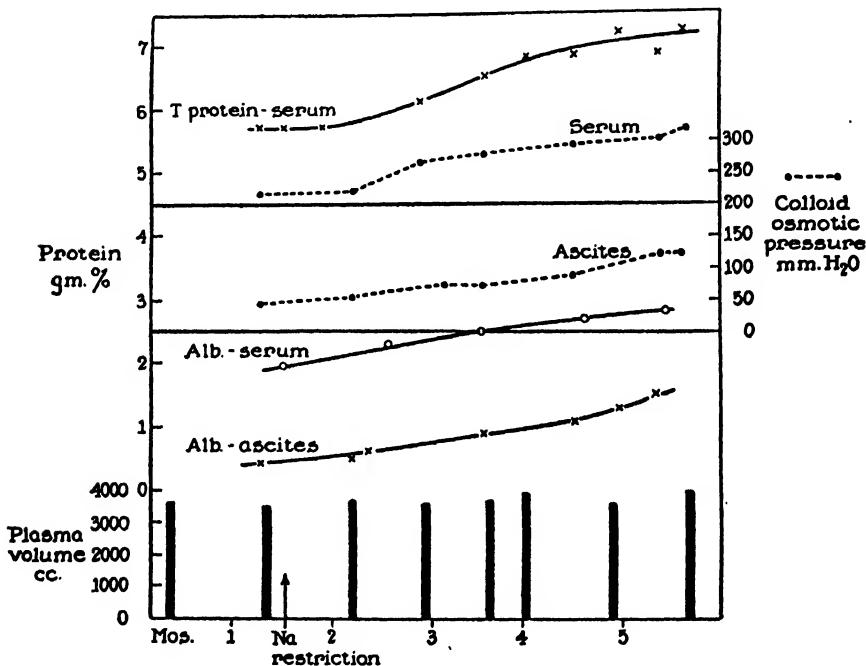
Fig. 5.—Rise in values for determinations of proteins and colloid osmotic pressure in serum and ascitic fluid following Na restriction.

enable them to excrete the increased Na and they did not reform ascites. These patients gradually absorbed the remaining ascitic fluid and returned to a normal life. Eight patients were still unable to excrete larger amounts of Na and accumulated ascites on the normal diet. These patients had not regressed during the three months of Na restriction and all of them appeared slightly improved but they had not been cured. Na restriction was reinstituted in these individuals. Three of these have since showed increased Na excretion representing loss of ascitic fluid after more than six months of therapy. Others in the group are still being treated with combined albumin, liver extract, and

low Na therapy. One of these patients died of esophageal hemorrhages. Patient 4 who did not tolerate the low Na diet also died.

In all of the patients the diet appeared to be beneficial in stopping the malignant course of events brought about by removal of protein through paracenteses in the already depleted individual, thus enabling dietary therapy to become effective. The long-term clinical results are difficult to evaluate but the fact that ascites formation could be completely controlled by rigid Na restriction was clearly evident.

SUMMARY

1. The results of rigid NaCl limitation in the diet of thirteen patients with cirrhosis of the liver and long-standing ascites are presented.

2. Ascites formation ceased for three months in twelve of the thirteen patients. Urine output increased in each case commensurate with decreased fluid retention.

3. Four patients did not reform ascites when a normal NaCl intake was tried after three months; eight patients did reform ascites.

4. Serum protein levels showed a rise following therapy. This is explained primarily on the basis of retention in the serum of protein which previously had been lost in the ascitic fluid.

5. A high protein and high caloric diet could be maintained despite rigid salt limitation.

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THE STABILIZATION OF SERUM LIPID EMULSIONS BY SERUM PHOSPHOLIPIDS

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Certain sera with marked elevations in total lipid concentration are completely clear, whereas other sera with small or only moderately elevated lipid content are grossly milky or lipemic. Since lipids exist in the serum as colloidal particles, the clarity or turbidity of serum will be determined by the size of these particles. Particles with diameters less than $\frac{1}{4}$ of the wave length of visible light (or 0.1μ) will not be seen in the visible light range and the containing serum will be clear, whereas larger particles by interrupting light rays give the serum the appearance of turbidity or lipemia. The purpose of the present study is to analyze the inherent qualities which make for invisible particle size of lipid droplets in clear sera and visible droplets in lipemic sera and to define the possible relationship of this problem to that of atherosclerosis.

Macheboeuf (1) in 1928 isolated a protein fraction rich in phospholipids and cholesterol esters which even in high concentration dissolved completely and transparently in water. He suggested that a lipid-protein combination might explain the manner of existence of lipid in clear serum, and his later work (2) has tended to implicate the protein, rather than the lipids, as the more fundamental determinant of lipid "solubility." However, Boyd (3) in 1937 observed a quantitative relationship between low serum phospholipid levels and lipemia. The present study demonstrates that sera with high total lipid concentration which contain predominant amounts of phospholipid are invariably clear and that in all sera tested the enzymatic removal of serum lecithin produces or increases lipemia. While the present data do not deny the importance of the lecithin-cholesterol-protein complex of Macheboeuf, they support the thesis that the concentration of serum phospholipids may be the limiting factor in determining the clarity of serum.

Materials and Methods

Patients whose sera provided the basis for this work were under study at the Hospital of The Rockefeller Institute for Medical Research. Clear high lipid sera were obtained from 16 patients with chronic partial intrahepatic biliary obstruction and cirrhosis, 13 of whom developed xanthomatous skin lesions (4) (hereafter referred to as "primary biliary cirrhosis"). Lipemic sera were for the most part from patients with the nephrotic syndrome.

Blood was drawn at least 12 hours after the last meal, allowed to clot, and the serum separated. Chemically clean glassware was used throughout; sterile precautions were not taken. Sera were analyzed chemically and enzymatically immediately, or after variable periods of

storage at 4°C. when it was found that storage for as long as 1 year did not alter materially the analyses discussed herein.

Total serum lipids were measured by a recently modified manometric lipid carbon method (5). Lipid phosphorus was determined by a modification of the Fiske and SubbaRow method (6), and lipid P was converted to phospholipid by the factor of 25. Free and total cholesterol were measured by the Schoenheimer-Sperry method (7). More recently, all major lipid components have been determined by these methods on a single alcohol-ether extract of a tungstic acid precipitate of 2 cc. of serum with neutral fat calculated by subtracting the lipid carbon of cholesterol and phospholipid from the total lipid carbon, as described in reference 5.

The lecithinase used in these experiments was the alpha-toxin of *Cl. welchii*. MacFarlane and Knight (8) in 1941 identified *Cl. welchii* alpha-toxin as an enzyme splitting lecithin into phosphorylcholine and diglyceride with pH optimum of 7.0 to 7.6, activated by Ca^{++} and inhibited by Ca^{++} precipitants and *Cl. welchii* antitoxin. The enzyme kinetics were further characterized by Zamecnik, Brewster, and Lipmann (9) in 1947, who concluded that lecithin was the specific substrate for the enzyme. In 1948 MacFarlane (10) brought forward evidence for slow enzymatic degradation of sphingomyelin as well as lecithin, but cephalins are stated by Zamecnik and coworkers (9) and by MacFarlane (10) to be unaffected. The enzyme has not been crystallized nor obtained in a pure form.

The concentrate of the clostridial culture used in these studies contained 200 M.L.D. of alpha-toxin per cc. as determined by intravenous injection in 13 to 14 gm. Rockefeller Swiss mice (11). Hereafter, for convenience, various "M.L.D.'s of toxin" will be understood to refer to appropriate dilutions of this concentrate. Moreover, no lipase activity was found in this concentrate when measured by hydrolysis of Tween 20 (12), or by titration of free fatty acids in ether extracts of serum after incubation with alpha-toxin. There were 570 units of hyaluronidase activity per cc., as measured viscosimetrically (13). For experimental use the enzyme concentrate was diluted with a calcium-rich gelatin solution buffered at pH 7.2 with borate as suggested by Adams (14). Lipid P was determined in such mixtures after trichloracetic acid precipitation and extraction with Bloor's alcohol-ether mixture. Acid-soluble P was measured on the trichloracetic acid supernate.

RESULTS

1. Lipid Patterns in Clear and Lipemic Sera.—Fig. 1 presents four pairs of abnormal serum lipid patterns with various lipid concentrations as contrasted to the pattern and lipid content in normal serum. The major component in each clear serum is seen to be phospholipid, whereas in lipemic nephrotic sera neutral fat predominates. Cholesterol fractions did not differ significantly in sera 2 and 3 or in sera 4 and 5, and in the other pairs the total cholesterol was similar. As an illustration of the lack of correlation between total lipid content and its physical state in the serum, it is seen that clear serum 8 contains almost three times as much lipid as milky serum 3. A larger series of data is presented in Table I.

Clear serum with elevated total lipid content has been found only in obstructive jaundice, whether intrahepatic or extrahepatic in origin (4, 15). The correlation between clarity and phospholipid predominance is seen in Fig. 2, where typical patterns of high lipid sera in a variety of abnormal conditions are presented. Only the high phospholipid serum of obstructive jaundice is clear; all other high lipid sera are milky.

In 25 normal sera, in which total lipids ranged from 440 to 766 mg. per cent, the ratio of phospholipid to total lipid (PL/TL) was found to have a maximum range of 0.31 to 0.40. In Fig. 3 the PL/TL of 19 nephrotic and 24 liver disease sera are plotted against their respective total lipid concentrations. All lipemic sera (solid symbols), regardless of total lipid content, fell below the normal range of PL/TL, whereas 21 of 24 clear sera (open symbols) were in the normal or high PL/TL range. The difference between the mean ratios of these two groups was statistically significant. Other serum lipid ratios are compared in Table II.

Comment.—On the basis of a large number of lipid patterns determined in a variety of diseases associated with elevated total lipids, the high phospholipid proportion in clear sera contrasted strikingly with the low phospholipid
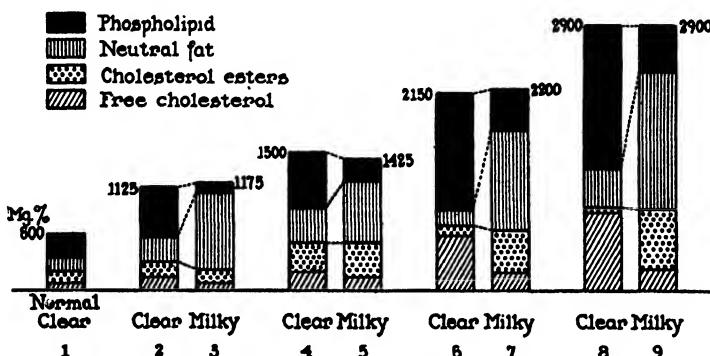
FIG. 1. Serum lipid patterns in normal and in selected pairs of sera with equally elevated total lipid concentration. Clear sera from patients with primary biliary cirrhosis; milky sera from nephrotic patients.

proportion in lipemic sera. Pertinent to this point is the report of Boyd and Connell (17) of a serum with total lipid of only 447 mg. per cent occurring in a patient with severe hepatitis in which the phospholipid content was only 52 mg. per cent; the serum was grossly lipemic.

2. Unsuccessful in Vitro Attempts at Clarification of Lipemic Serum.—If the PL/TL ratio is of importance in maintaining the clarity of serum, the addition of clear high phospholipid serum to lipemic low phospholipid serum might result in clarification.

In four experiments aliquots of clear high lipid serum and clear normal lipid serum were mixed under sterile conditions with aliquots of lipemic nephrotic serum in various proportions from 1:1 to 10:1, and held for 24 to 48 hours at 25 and 37°C. There was no change in turbidity of these mixtures as measured in a Coleman, Jr., spectrophotometer at 650 mμ.

Comment.—In regard to these unsuccessful attempts to clarify lipemic serum, it is of interest that a number of laboratories have recently produced triglyc-

TABLE I

Serum Lipid Patterns of 36 Lipemic and Clear Sera Paired According to Total Lipid Content

Serum No.	Lipemic or clear	Total lipid	Total phospholipid	Neutral fat	Total cholesterol	Free cholesterol	Free/total[*] cholesterol ratio	Phospholipid[*] / Total lipid ratio	Phospholipid[*] / Total cholesterol ratio
		mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*			
Normal mean ±S.D.‡	Clear	613 ±74	219 ±22	130 ±43	185 ±24	56 ±7	0.30 ±0.02	0.31–0.40	1.0–1.3
1	Lipemic	1128	312	62	518	136	0.26	0.28	0.60
2	Clear	1165	394	270	385	150	0.39	0.34	1.02
3	Lipemic	1144	240	352	383	111	0.29	0.21	0.63
4	Clear	1138	325	387	312	100	0.32	0.29	1.04
5	Lipemic	1230	342	266	430	121	0.28	0.28	0.56
6	Clear	1236	635	63	437	304	0.70	0.51	1.45
7	Lipemic	1235	294	367	400	119	0.30	0.24	0.74
8	Clear	1259	356	523	279	132	0.47	0.28	1.28
9	Lipemic	1377	216	392	525	131	0.25	0.16	0.41
10	Clear	1366	431	318	435	141	0.33	0.32	0.99
11	Lipemic	1497	354	287	591	164	0.28	0.24	0.60
12	Clear	1514	688	351	402	284	0.71	0.45	1.71
13	Lipemic	1618	351	419	591	186	0.31	0.22	0.59
14	Clear	1632	744	357	459	343	0.75	0.46	1.62
15	Lipemic	1618	270	466	591	133	0.23	0.17	0.46
16	Clear	1601	547	465	483	313	0.65	0.34	1.13
17	Lipemic	1695	414	82	850	289	0.34	0.24	0.49
18	Clear	1686	769	184	571	311	0.54	0.46	1.34
19	Lipemic	1700	480	314	625	171	0.27	0.28	0.77
20	Clear	1738	863	178	625	493	0.79	0.50	1.38
21	Lipemic	1729	462	216	725	201	0.28	0.27	0.64
22	Clear	1752	869	207	600	478	0.80	0.50	1.45

* Ratio on weight basis.
‡ Ahrens, Eder, and Van Slyke (5).

TABLE I—*Concluded*

Serum No.	Lipemic or clear	Total lipid	Total phos-pholipid	Neutral fat	Total choles-terol	Free choles-terol	Free/total* cholesterol ratio	Phospholipid* / Total lipid ratio	Phospholipid* / Total cholesterol ratio
		mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*			
23	Lipemic	1752	336	535	608	168	0.28	0.19	0.55
24	Clear	1743	688	440	516	357	0.69	0.40	1.33
25	Lipemic	1997	330	880	567	211	0.37	0.17	0.58
26	Clear	2034	1088	173	646	442	0.69	0.63	2.00
27	Lipemic	2198	444	813	653	189	0.29	0.20	0.68
28	Clear	2232	1350	54	650	524	0.81	0.61	2.08
29	Lipemic	2270	345	786	800	254	0.32	0.15	0.43
30	Clear	2248	1088	245	625	493	0.79	0.48	1.74
31	Lipemic	2332	480	833	710	212	0.30	0.21	0.68
32	Clear	2384	1230	185	860	685	0.80	0.52	1.43
33	Lipemic	2712	430	1423	616	196	0.32	0.16	0.70
34	Clear	2680	1331	418	750	604	0.85	0.50	1.77
35	Lipemic	2910	501	1116	891	243	0.27	0.17	0.56
36	Clear	2969	1400	632	838	679	0.81	0.47	1.67

eride emulsions for intravenous alimentation of patients using a variety of agents as stabilizers, such as human serum albumin (18), gelatin (18), soy bean phosphatides (19), and surface-active agents like Span 20 (20) and monoglycerides (21). Although creamy emulsions with particle size not exceeding 1.5 μ have been made, a completely transparent emulsion of hydrophobic lipids such as is seen in the serum of obstructive jaundice has never been duplicated.

3. In Vitro Production of Lipemia in Clear Serum.—If the PL/TL ratio is of importance in maintaining the clarity of serum, the removal of phospholipids from clear serum might be expected to result in lipemia. This has been accomplished successfully by enzymatic degradation of serum lecithin by *Cl. welchii* alpha-toxin. This enzymatic cleavage was of particular suitability to the present investigation since the strongly polar and hydrophilic phosphoryl-choline group which might act as a dispersing agent for the hydrophobic lipids was split from the lecithin molecule. Although there is evidence that sphingo-myelin as well as lecithin is hydrolyzed by this enzyme (10), the rate of hy-drolysis of these two choline-containing phospholipids in serum has not been
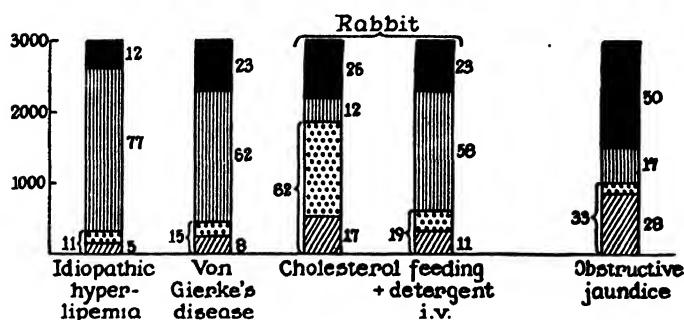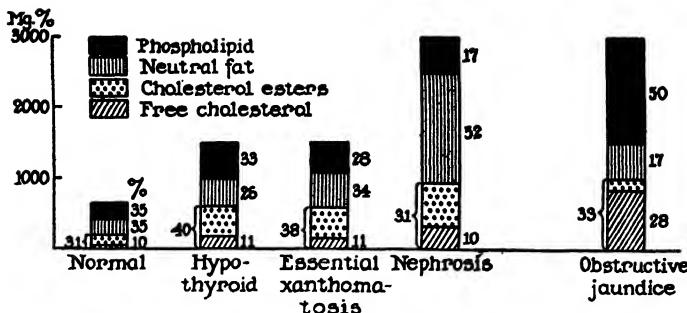
FIG. 2. Representative serum lipid patterns in a variety of conditions associated with elevated serum lipids. All sera are lipemic except that found in obstructive jaundice (acute or chronic intra- or extrahepatic obstruction). Hypothyroid pattern from data of Gildea, Man, and Peters (16).
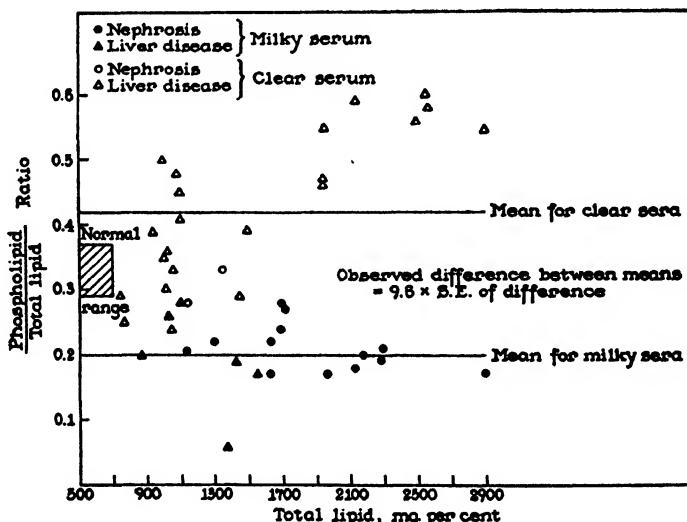
FIG. 3. Correlation between total lipid concentration, lipemia or clarity, and phospholipid/total lipid ratio in primary biliary cirrhosis and nephrosis. The range of normal is found in the hatched area at the left.

compared and is not the subject of this report. Hereafter, for convenience, "lecithin" will signify those phospholipids which are hydrolyzed by *Cl. welchii* alpha-toxin.

TABLE II

Relation of Serum Lipid Components to Serum Milkiness

Ratio	Normal range	Milky sera*	Clear sera‡
Phospholipid: total lipid	0.29–0.37	0.20 ± 0.04	0.42 ± 0.10
" : neutral fat	1.1 –2.0	0.41 ± 0.21	3.44 ± 3.39
" : total cholesterol	1.0 –1.3	0.82	1.25
Cholesterol esters: total lipid	0.17–0.24	0.22	0.17
Free cholesterol: total lipid	0.10–0.16	0.10	0.19

All ratios expressed on basis of weight.
* Mean of 19 sera ± S.D.
‡ Mean of 25 sera ± S.D.

TABLE III

*Effect of Cl. welchii Alpha-Toxin on Soy Bean Lecithin and Soy Bean Cephalin**

Substrate		Enzyme content	Final volume of buffered mixture	Flocculation after 3 hrs. at 37°C.
Concentration	Amount			
mg. per cent	*cc.*	*M.L.D.*	*cc.*	
A. Lecithin				
250	0.1	8	2.6	++
500	"	"	"	+++
1000	"	"	"	++++
2000	"	"	"	++++
B. Cephalin				
250	"	"	"	0
500	"	"	"	0
1000	"	"	"	0
2000	"	"	"	0

* Fractionated from crude soy bean phosphatides (asolectin) according to Levene and Rolf (22).

It was found that, when 0.1 cc. of serum was incubated at 37°C. with 8 M.L.D. of toxin in 2.4 cc. of buffer, the clear mixture became lipemic within 15 minutes with linear increase in optical density which reached a nearly maximal value within 2 hours in all types of sera, with very small increments thereafter for many hours. Since the initial total phospholipid concentration of the sera tested varied from 150 to 2,000 mg. per cent, it is seen that, under the conditions stated, 8 M.L.D. of toxin was exposed to substrate concentrations of only 6 to 80 mg. per cent. (Zamecnik and coworkers (9) have shown that 9 M.L.D. of enzyme is saturated with

substrate at a 2.6 gm. per cent lecithin concentration.) Increases in turbidity were noted in all sera tested regardless of total lipid concentration and whether initially lipemic or clear.

That these changes in turbidity were in fact due to lecithinase action rather than to some unrecognized component in the toxin concentrate is substantiated by the chemical studies detailed below which relate phospholipid breakdown to turbidity change; by the acceleration of the phenomenon by Ca^{++} and Mn^{++} and retardation by Cu^{++} and Fe^{+++}, in line with the kinetic studies of MacFarlane and Knight (8) and Zamecnik, et al. (9); and by the production of flocculation in aqueous solutions of soy bean lecithin but not of soy bean cephalin, as detailed in Table III. In regard to the possible action of the hyaluronidase contained in the concentrate, it was found that marked changes in turbidity were not accompanied by changes in viscosity, as measured at 37.0°C. in Ostwald viscosimeters, using 3 cc. of high lipid serum and 8 M.L.D. of enzyme in 1 cc. of buffer.

1:2 Dilution of Serum:Saline   1:25 Dilution of Serum: Lecithinase Solution
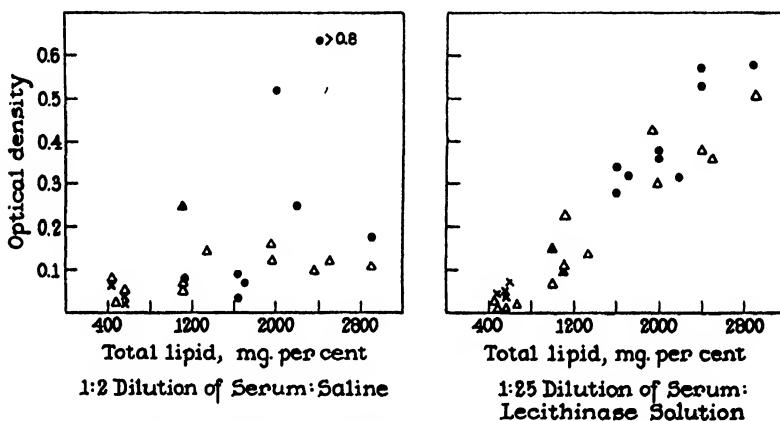
FIG. 4. (Left). Lack of correlation between total lipid content and the optical densities of sera diluted 1:2 with saline. Readings at 650 mμ in small cuvettes, water blank. Symbols as in Fig. 3. x = normal sera.

(Right). Correlation between total lipid content and optical densities of 1:25 dilutions of sera incubated with Cl. welchii lecithinase for 2 hours at 37°C. Readings at 650 mμ in small cuvettes, blanks of 1:25 dilution of same sera without added enzyme.

Utilizing the testing procedure outlined above, a variety of clear and lipemic sera were incubated with lecithinase. Fig. 4 (right) shows that at 2 hours there was a linear relationship between total lipid concentration and optical density in 28 sera, although the optical densities of these sera prior to enzymatic hydrolysis gave no indication of total lipid content (Fig. 4, left). Also, it will be noted from the serum dilutions used in the two groups of tests that there was more than a tenfold increase in optical density of the enzymatically treated sera as compared to the untreated aliquots. Darkfield microscopic examination during the course of this enzymatic hydrolysis showed the gradual appearance of discrete particles with Brownian movement. At the end of the reaction, and even after standing for 24 hours, the particles remained discrete and no larger than 1 μ in diameter and continued to show Brownian movement.

They were indistinguishable from the chylomicrons of alimentary or nephrotic lipemia.

In attempts to demonstrate possible differences between clear and lipemic sera with regard to their reaction to lecithinase, decreasing amounts of enzyme were added to several aliquots of serum while holding the volume relationships constant. Fig. 5 demonstrates that with decreasing enzyme concentration the onset of turbidity change is progressively delayed, although if sufficient incubation time is allowed, the final optical densities are almost the same. It is of interest that similar families of curves were obtained under these con
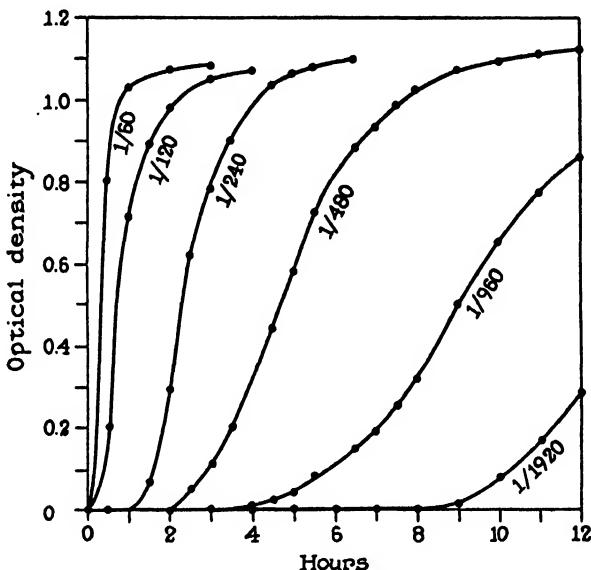
Fig. 5. Serum aliquots incubated with increasing dilutions of concentrated *Cl. welchii* lecithinase preparations at 37°C. (1 cc. serum + 1 cc. buffered enzyme).

ditions regardless of initial serum lipid composition, both in the lipemic sera of nephrotics and in all clear sera, but not in a extremely lipemic sera from a patient with idiopathic familial hyperlipemia (see below).

Comment.—Loss of the hydrophilic group of "lecithin" appears to destroy the stabilizing effect of this phospholipid upon the hydrophobic lipids. Destruction of "lecithin" initiates lipemia in clear sera with PL/TL of 0.29 or more and increases lipemia in turbid sera with PL/TL less than 0.29. "Lecithin" is, therefore, partially effective as a stabilizer even in lipemic sera with relatively small phospholipid fractions.

4. Correlation between Phospholipid Breakdown and Turbidity.—In Fig. 5 it is noted that the onset of change in optical density can be greatly delayed if the enzyme concentration is sufficiently low. The conversion of lipid P to

acid-soluble P during this lag period was determined in order to correlate phospholipid breakdown with optical density changes. Over a wide range of total lipid concentration the quantity of enzyme was determined which would allow an approximately 2 hour lag period before a change in optical density of the serum-enzyme mixture was detectable.

In these experiments several 0.5 cc. aliquots of serum were diluted with 10.0 cc. of buffered enzyme solution in large cuvettes, in order that at intervals the optical densities could be measured spectrophotometrically against a similarly diluted aliquot without enzyme. It was
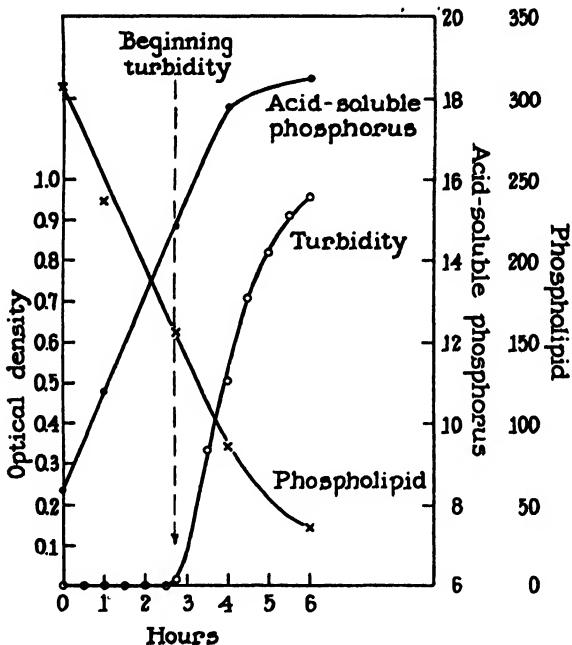
Fig. 6. Conversion of lipid P to acid-soluble P by *Cl. welchii* lecithinase at a linear rate prior to onset of turbidity in a clear serum with 1040 mg. per cent total lipid and 300 mg. per cent phospholipid (1.2 M.L.D. enzyme per 0.5 cc. serum).

found necessary for these purposes to use from 5 M.L.D. of enzyme per 0.5 cc. of normal sera to 0.6 M.L.D. per 0.5 cc. of sera containing up to 2500 mg. per cent total lipid, a paradoxical relationship explained by the extreme dilution of substrates. At certain intervals relative to turbidity change the reaction was stopped with trichloracetic acid, and measurements made of acid-soluble and lipid P.

Fig. 6 illustrates a representative experiment in which a 2½ hour lag period was produced prior to the onset of turbidity, during which time there was a linear decrease in lipid P and a corresponding increase in acid-soluble P. Similar curves of phospholipid destruction prior to onset or increase in turbidity were demonstrated in this manner in the lipemic sera of 3 nephrotic patients as well as in the clear high lipid sera of 18 patients with biliary cirrhosis.

Similar testing of intensely lipemic sera from a patient with idiopathic familial hyperlipemia (whose lipid pattern is shown in Fig. 2) showed that turbidity change began at zero time even when extremely low enzyme concentrations were used. Thus, no lag period could be produced when 0.5 cc. aliquots of this serum were incubated with 10 cc. of buffered toxin containing from 40 M.L.D. to 0.5 M.L.D. of *Cl. welchii* lecithinase. Fig. 7 shows that phospholipid breakdown resembled that in Fig. 6 but that increases in optical density and phospholipid hydrolysis proceeded hand in hand.
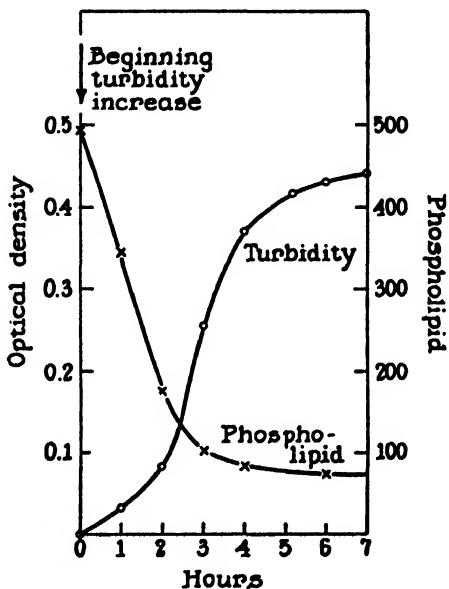
FIG. 7. Hydrolysis of phospholipid by *Cl. welchii* lecithinase with immediate increase in turbidity in serum of idiopathic hyperlipemia with 3000 mg. per cent total lipid and 360 mg. per cent phospholipid (1 M.L.D. per 0.5 cc. serum).

Comment.—Under these conditions significant enzymatic destruction of "lecithin" was demonstrated prior to change in optical density both in clear and in lipemic nephrotic sera. This suggests that, although part of the lipids in nephrotic serum occurs in particles of large enough size to be visible, a significant portion is present as particles of invisible size. As phospholipid is gradually destroyed, these invisible particles gradually coalesce until they reach visible size, with a resulting lipemia proportional to total lipid content. However, in the serum of a patient with idiopathic hyperlipemia, enzymatic removal of "lecithin" resulted in an immediately perceptible increase in optical density, suggesting that the *in vivo* lipid particle size was nearly maximal and that "lecithin" stabilization was relatively unimportant.

DISCUSSION

Boyd (3) in 1937 in a statistical study of 36 non-fasting norm l sera found that lipemic sera contained a higher proportion of neutral fats an did clear sera. He suggested that lipemia might be due to a relative insuli ncy of phospholipids. The present data on the clarity or milkiness of f g sera in a variety of disease states lend the weight of experimental eviden this hypothesis.

Early reports suggested that visible lipid droplets in s coated with protein (23, 24). Elkes, Frazer, Schulman, and Stewart 15 reported the adsorption of various serum protein fractions at l oil-water interface with flocculation of these coated particles at ined by the isoelectric point of the protein used. With 2.5 mg. or more oi protein per square meter of interfacial area there was maximum emulsion stability with lipid droplets remaining discrete and non-coalescent. From their data it can be calculated that a lipemic serum with total lipid content of 5000 mg. per cent (about 10 times normal) would require approximately 150 mg. per cent of protein at the oil-water interface, if all lipid was present droplets of 0.5 μ diameter. Under these conditions, the amount of protein a abl n serum for forming such a layer would not appear to be the limiting facto forma- tion of stable lipid emulsions. Indeed, even in nephrosis with severe hypoproteinemia, with elevated serum lipids and severe lipemia, lipid particles remain discrete and do not coalesce, indicating that there is sufficient protein present to form a layer at the interface and therewith a stable lipid emulsion.

Macheboeuf's extensive studies (2) on lipoproteins have resulted in the separation at acid pH of lipid-protein complexes which go into completely transparent water solution at neutral pH. These complexes, which Macheboeuf has termed *"cénapses,"* contain only cholesterol esters and phospholipids, without triglycerides or free cholesterol, and are 50 to 60 per cent protein. His studies raise a question which cannot be satisfactorily answered at present; namely, whether clarity or lipemia of serum may depend not only on the quantity of phospholipid present but also on the character and amount of the protein to which lipids appear to be firmly attached. However, in the present authors' laboratory a procedure has been developed (26) by which lipids can be completely separated from the serum without altering the electrophoretic characteristics of the remaining proteins. Analyses of the lipid pellicle centrifuged to the surface have shown approximately 30 per cent tightly bound protein in normal and nephrotic lipemic sera and only about 15 per cent protein in clear high lipid sera of biliary cirrhosis. Under these conditions the amount of protein tightly bound to lipid was apparently unrelated to clarity or milkiness of serum.

Nagler (27) in 1939 noted that *Cl. welchii* toxin produced an opalescence in

some sera, and used this reaction for the titration of antitoxin. In 1941 Mac-Farlane and Knight (8) demonstrated that the Nagler reaction was produced by enzymatic hydrolysis of lecithin and that alpha-toxin was in fact lecithin-ase. The kinetics of the enzyme reaction were further defined for human serum (28) and a preliminary report of the degradation of human serum lipoprotein by a related lecithinase has also appeared (29). In a brief note Elkes and Frazer (30) in 1943, observing the Nagler effect of *Cl. welchii* on serum, suggested that phospholipids may stabilize fat droplets in serum. The present studies demonstrate that, under suitable conditions and in all sera tested, lipid particles coalesce and reach visible size after hydrolysis of "lecithin" by *Cl. welchii* lecithinase, that the resultant turbidity is proportional to total lipid content, and that there is a definite relationship between phospholipid breakdown and production of turbidity. These results seem entirely in keeping with the hypothesis suggested by MacFarlane and Knight in 1941 (8), that fat is liberated from combination with lipoprotein and that "creaming" is due to the decomposition of "lecithin" which acts as a stabilizing agent in lipid-protein emulsions.

The surface-active properties of lecithin have been known for many years. In 1923 Seifriz (31) demonstrated that lecithin stabilizes oil-in-water emulsions. From studies of monomolecular films of lecithin Leathes in 1925 (32) concluded that the hydrophilic phosphorylcholine group reduced the cohesive force which the two fatty acid radicals exerted upon each other, causing each radical to occupy twice its usual area. On the basis of these and the present data, it may be suggested as a working hypothesis that lecithin finds itself at the oil-water interface of the serum lipid droplet where it forms an undetermined type of bond with the protein film, enmeshing cholesterols and triglycerides in the expanded hydrophobic fatty acid portion of its molecule by Van der Waals forces.[1] It may follow that the greater the concentration of lecithin, the larger the total oil-water surface area which it can stabilize; hence, with a given concentration of total serum lipid, the greater the lecithin concentration, the smaller the lipid particle which may exist.

The possible additional effect of bile salts in producing clarity in the high lipid sera encountered in biliary obstruction cannot be assessed at present, since reliable methods for the accurate measurement of bile salts are not yet available. Although the present evidence appears to implicate phospholipids as the key factor in serum lipid emulsification, the possibility that bile salts may play a part cannot be entirely overlooked.

Finally, the implications of differences in serum lipid pattern in the pathogenesis of tissue lipid deposition, especially atherosclerosis, deserve comment.

[1] That this is an oversimplification of a complex colloidal phenomenon can be seen by consulting three recent reviews by surface chemists (33–35).

A strikingly high incidence of premature atherosclerosis has been noted in patients with nephrosis, hypothyroidism, and essential xanthomatosis, where the phospholipid/cholesterol ratio in serum is less than 1 (Fig. 2). On the other hand, atherosclerosis has not been reported in the few autopsied cases of von Gierke's disease or in idiopathic hyperlipemia, where neutral fat levels are greatly elevated but the phospholipid/cholesterol ratio is more than 1. Indeed, the presence of normal aortas in patients who for years have marked lipemia fails to substantiate Moreton's hypothesis (36) that serum lipemia *per se* may initiate atheromatosis. The possible rôle of an intracellular lipemia due to intracellular lecithinase (37) in the genesis of atheromatosis has not been investigated.

In 16 patients with primary biliary cirrhosis and xanthomatous infiltration of the skin (4), clinical, electrocardiographic, and x-ray evidence of atheromatous disease has been lacking. In four of these patients who have been autopsied the degree of atherosclerosis was not greater than that to be expected at their ages, although skin xanthomatosis was severe. In all patients of this group the phospholipid/cholesterol ratio was more than 1 (15). In experimental studies Kellner, Correll, and Ladd (38, 39) have been able to decrease the incidence of atherosclerosis in rabbits by injecting surface-active agents (Tween 80 and Triton A20) intravenously, a procedure which markedly increased the phospholipid/cholesterol ratio of serum (Fig. 2). Thus, on the basis of observations in a variety of disease states and in experimental studies, a relationship appears to exist between the fixation of lipid in intimal cells and decreased phospholipid/cholesterol ratios.

CONCLUSIONS

Clarity of high lipid sera is closely correlated with elevated proportions of serum phospholipids, and lipemia (milkiness) with low proportions of phospholipids. Clear high lipid sera occur uniquely in obstructive jaundice, both intra- and extrahepatic in origin.

Destruction of the polar nature of serum "lecithin" by enzymatic hydrolysis, using *Cl. welchii* lecithinase, results in a degree of lipemia which is linearly proportional to total lipid content in clear or lipemic high lipid or normal lipid sera.

Even in grossly lipemic sera, a significant proportion of the serum lipids is masked in particles of invisible size. Enzymatic removal of the stabilizing properties of serum "lecithin" unmasks this hitherto invisible fraction.

The concentration of serum phospholipids available for complex formation with serum proteins appears to be an important factor in determining particle size of serum lipids and hence of their occurrence in serum as masked or as visible particles.

The implications of these findings for studies of the genesis of atheromatosis are discussed.

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THE RELATIONSHIP BETWEEN SERUM LIPIDS AND SKIN XANTHOMATA IN EIGHTEEN PATIENTS WITH PRIMARY BILIARY CIRRHOSIS

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Since 1938 several reports have appeared in the literature establishing xanthomatous biliary cirrhosis as a specific disease (1–5). Thannhauser, while supporting this concept, has altered his original contention regarding the primary role of essential xanthomatosis in the causation of biliary obstruction and secondary biliary cirrhosis (3, 5). It is now generally agreed that the xanthomatosis of this disease is secondary to a type of biliary cirrhosis. Observations on 18 female patients to be discussed in this paper support this conclusion. In addition, evidence is presented for the existence in the development of the full-blown disease of a pre-xanthomatous stage which is characterized by a lesser degree of biliary obstruction and a less impressive elevation of the lipids of the serum. Xanthomata appear only when the total lipid elevation is extreme and prolonged, and the disease frequently arrests itself prior to the development of xanthomata. Thus, a level of total serum lipids critical for the appearance of xanthomata can be demonstrated. Data concerning the typical lipid pattern of the disease based on a newly available method of total lipid estimation are presented (6).

Material

Sera which form the basis of this study were obtained from 18 female patients with chronic partial intrahepatic biliary obstruction and cirrhosis (hereafter termed "primary biliary cirrhosis"). Fifteen of these patients had xanthomatous skin lesions; in seven the lesions were generalized large tuberous and flat xanthomata; in eight the distribution was localized to the eyelids (hereafter called xanthelasma). Three of the 18 patients have not yet developed xanthomata. Thannhauser's classification (2) of these various types of xanthomata has been used in this report.

Clinical and laboratory data relevant to the singleness of the disease entity suffered by these 18 patients will be presented separately (7). Briefly, diagnostic criteria of primary biliary cirrhosis included the following: pruritus and prolonged painless jaundice without history, signs, symptoms, or operative findings of extrahepatic biliary obstruction or infection; sudden or insidious onset without known nutritional, toxic, or infectious hepatitis background; excellent physical condition and strength on a complete diet; appearance of xanthomata after months or years of jaundice and itching; pronounced liver enlargement but variable splenomegaly; no ascites, edema or evidence of portal hypertension except as late signs; rare spiders and liver palms;

melanosis and thick dry skin. Laboratory data included: signs of   truction
(severe steatorrhea; elevated bilirubin, alkaline phosphatase and   ls; clear
serum; decreased fecal urobilinogen, increased urine bile and uro'   ıdequate
protein synthesis (minor depression of serum albumin, normal ı·   ı, normal
or elevated fibrinogen, normal or slightly decreased serum esterase); elevated gamma
globulin; cephalin flocculation 0 to 4+; moderate normochromic-normocytic anemia.

Methods

Total serum lipids were determined by the manometric lipid carbon method of
Ahrens, Eder and Van Slyke (6). Analysis is made of lipid carbon in tl   'cohcl-ether
extract of a tungstic acid precipitate of serum, a procedure which has , en f ,nd to
avoid contamination of the final extract by non-lipid impurities and which ·   ;ures
95% or more of the lipid. Lipid P was measured by a modification of the Fiske and
SubbaRow (8) method, and convertd to phospholipid by the factor of 25. 'l otal and
free cholesterol were determined by the Schoenheimer-Sperry method (9). Neutral fat
was calculated by difference between the total lipid carbon and that of cholesterol and
phospholipid as described in (6). In the last three years more than 300 serum lipid
patterns have been carried out at frequent intervals in the present series of patients.
In addition, a turbidimetric procedure (10, 11) for rapid estimation of total serum
lipids has been extremely useful as a screening procedure and as a means of following
the course of serum lipids in patients examined at frequent intervals.

Serum cephalin was calculated from the lipid P unhydrolyzed by *Cl. welchii* leci-
thinase, since MacFarlane (12) and Zamecnik, Brewster and Lipmann (13) have
shown that phosphatidylserine and phosphatidylethanolamine (the two known serum
*cephalins) are not affected by that enzyme. One-half cc. of serum was incubated at
37° C. for 24 hours with an excess of buffered enzyme, at which time the remaining
lipid P was measured in an alcohol-ether extract of the trichloracetic-acid-precipitated
serum, as described in (14). Lipid P was converted to cephalin by a factor of 23.5,
assuming equal concentrations of phosphatidylserine and -ethanolamine. The "ceph-
aline" values given below are recognized to be approximate and on the high side,
since no effort was made to maintain pH at optimal levels to compensate for increasing
acidity with hydrolysis, nor has it been conclusively proven that sphingomyelin is
entirely hydrolyzed under these conditions. It can be stated with assurance, however,
that cephalin levels were not higher than those listed.

RESULTS

Typical patterns of the serum lipids in eight different patients with primary
biliary cirrhosis are presented in Figure 1 along with the duration in each of
jaundice, xanthelasmic streaks in eyelids, or severe generalized xanthomata
(tuberous or flat types). These eight patterns show a marked similarity irre-
spective of total lipid concentration or of the presence or absence of xanthomata.
Preponderance of phospholipids is noted at all levels of total lipid concentra-
tion. The rise in free cholesterol concentration is also evident, although not
as marked as the phospholipid increase. Cholesterol ester concentration has
been normal or slightly elevated in all sera tested, except terminally when the

esterifying ability of the liver fails along with many other functions. However, the ratio of free to total cholesterol has been markedly elevated because of the characteristic increase in the free form. Neutral fat levels have been moderately elevated in all patients except during periods of malnourishment on low fat diets or terminally. Every fasting serum encountered in the present series of patients has been clear.

Figure 2 shows typical lipid patterns in other hyperlipemic conditions in order to demonstrate the uniqueness of the pattern in primary biliary cirrhosis. All high-lipid sera, with the exception of that found in biliary obstruction, are lipemic.

Mg.%

Phospholipid
Neutral fat
Cholesterol esters
Free cholesterol

3200, 2800, 2400, 2000, 1600, 1200, 800, 400, 0

Normal 600   1079   1340   1650   1848   2138   2500   2897   3145

	Jaundice	Xanthelasma	Severe xanthomata
	0	0	0
	2½	0	0
	1¼	4	0
	1	0	0
	3½	1¼	1½
	3½	1¾	1¾
	6	3	5
	3	2	2
	2¼	¼	¼

Duration, years
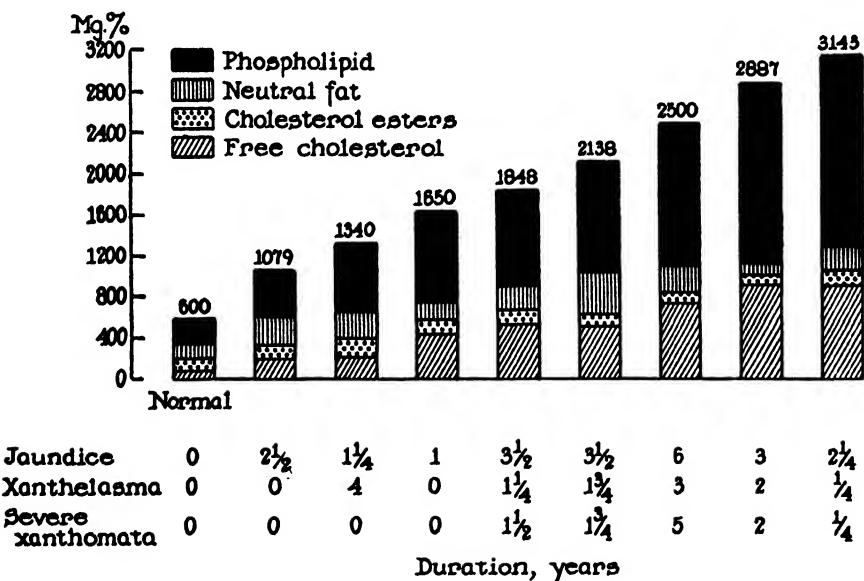
FIG. 1. Serum lipid patterns in eight patients with primary biliary cirrhosis.

Table I lists the total phospholipid and cephalin concentrations in 19 sera of patients with unexplained biliary cirrhosis as compared to five normals. Since it is seen that the cephalin fraction is very little increased in this disease, either lecithin or sphingomyelin or both must make up the characteristically elevated phospholipid fraction. Using other methods MacMahon and Thannhauser (5) report that in two of their patients, where individual phospholipids were determined, the phospholipid increase was mainly lecithin.

Figure 3 charts 150 separate serum lipid patterns determined in the present series at various stages of disease. Concentrations of the major lipid components are charted on the ordinate against the total lipid concentration of each serum on the abscissa, and for comparative purposes a line has been drawn through the phospholipid and cholesterol points. The range of normal, as determined
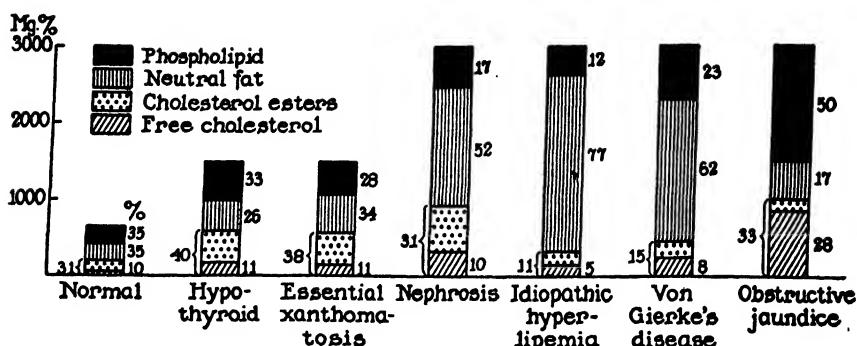
FIG. 2. Representative lipid patterns in various hyperlipemic conditions. All high-lipid sera are lipemic (milky) except that of obstructive jaundice (intra- or extra-hepatic in origin). Hypothyroid pattern calculated from data of Gildea, Man, and Peters (26).

TABLE I

	Serum no.	Total lipid	Total phospho-lipid	"Cepha-lin"	Total choles-terol	Free choles-terol	Free / total cholesterol	Neutral fat
		mg. %	mg. %	mg. %	mg. %	mg. %		mg. %
Normals	1	591	185	43	217	54	.25	88
	2	640	190	29	212	64	.30	116
	3	650	190	27	200	62	.31	174
	4	700	210	29	205	60	.29	201
	5	720	215	41	207	64	.31	209
Primary biliary cirrhosis	1	758	238	32	208	66	.32	224
	2	1000	386	55	325	129	.40	167
	3	1193	406	53	463	161	.35	126
	4	1230	363	53	432	138	.32	242
	5	1269	494	53	425	146	.35	177
	6	1355	425	80	387	164	.42	404
	7	1385	581	56	442	275	.62	258
	8	1615	844	75	583	443	.76	102
	9	1641	706	58	416	276	.66	432
	10	1678	900	59	608	443	.73	68
	11	1754	769	47	571	311	.54	253
	12	1927	912	65	675	536	.79	253
	13	1933	963	52	800	630	.78	64
	14	1943	995	53	566	450	.79	355
	15	2173	1110	93	691	528	.76	271
	16	2346	1350	82	650	524	.81	268
	17	2693	1525	94	866	756	.88	224
	18	3017	1688	108	1070	880	.82	141
	19	3027	1538	112	883	821	.93	568

in this laboratory in 20 sera, is shown in the hatched areas on the left. Patients who have not yet devleoped xanthomata or who have xanthelasma without other xanthomata are represented by open circles, whereas those who have or have had tuberous or flat xanthomata are shown by closed circles.

Several points are apparent from inspection of Figure 3. *1*) With increasing total lipid levels the predominant component is phospholipid rather than cholesterol or neutral fat. *2*) Free cholesterol is elevated in all sera but rises with increasing total lipids less sharply than phospholipids. *3*) Neutral fat is elevated in almost all sera, but less so than phospholipids and free cholesterol. *4*) All patients with total lipids of 1800 mg.% or more had severe xanthomata. *5*) Serum lipid patterns of pre-xanthomatous patients were indistinguishable from many of the patterns found in xanthomatous patients, as noted in the zone below 1800 mg.% total lipid.

Certain additional information could not be included in Figure 3. *1*) Patients with minor degrees of skin xanthomata (xanthelasma) had total lipid levels of about 1300–1800 mg.%. *2*) Patients without any xanthomata had total lipid levels of about 900–1300 mg.%. *3*) Elevated total lipid levels were found in all patients at some time in their course. The patient with the least elevation had total lipids in the normal range at certain intervals. *4*) The disappearance or diminution of xanthomata in four cases was preceded in all by dramatic spontaneous decrease in total lipid concentration. Patterns made periodically during these decreases account for the presence of closed symbols in Figure 3 below 1800 mg.% total lipid. They conformed in all respects to the patterns of patients in the pre-xanthomatous group.

The length of time required for the deposition of skin xanthomata in the presence of elevated serum lipids cannot be accurately stated, since in none of the patients could the initial rise in lipids be dated. In one pre-xanthomatous patient with total lipids of more than 2000 mg.%, severe generalized skin xanthomatosis was predicted and indeed developed floridly four months later (Figure 6). In another patient generalized flat xanthomata developed nine months after elevated serum lipids were recognized, and were similarly predicted in the pre-xanthomatous phase of her illness. A third patient developed flat xanthomata of the palms within three months of the onset of her illness, but only after nine months with total lipids consistently above 2000 mg.% did generalized tuberous xanthomata appear (Figure 5). Thus, it seems that severe xanthomata appear only after prolonged periods with marked serum lipid elevation, and in this there is considerable individual variation.

Figure 4 represents the rough correlation in this series of patients between total lipid and total serum bilirubin levels, both of which are regarded in this Hospital as indications of biliary obstruction (whether mechanical or purely functional retention cannot be stated with the evidence at hand). It is seen again that xanthomata are absent in the patients who are the least jaundiced

FIG. 3

530
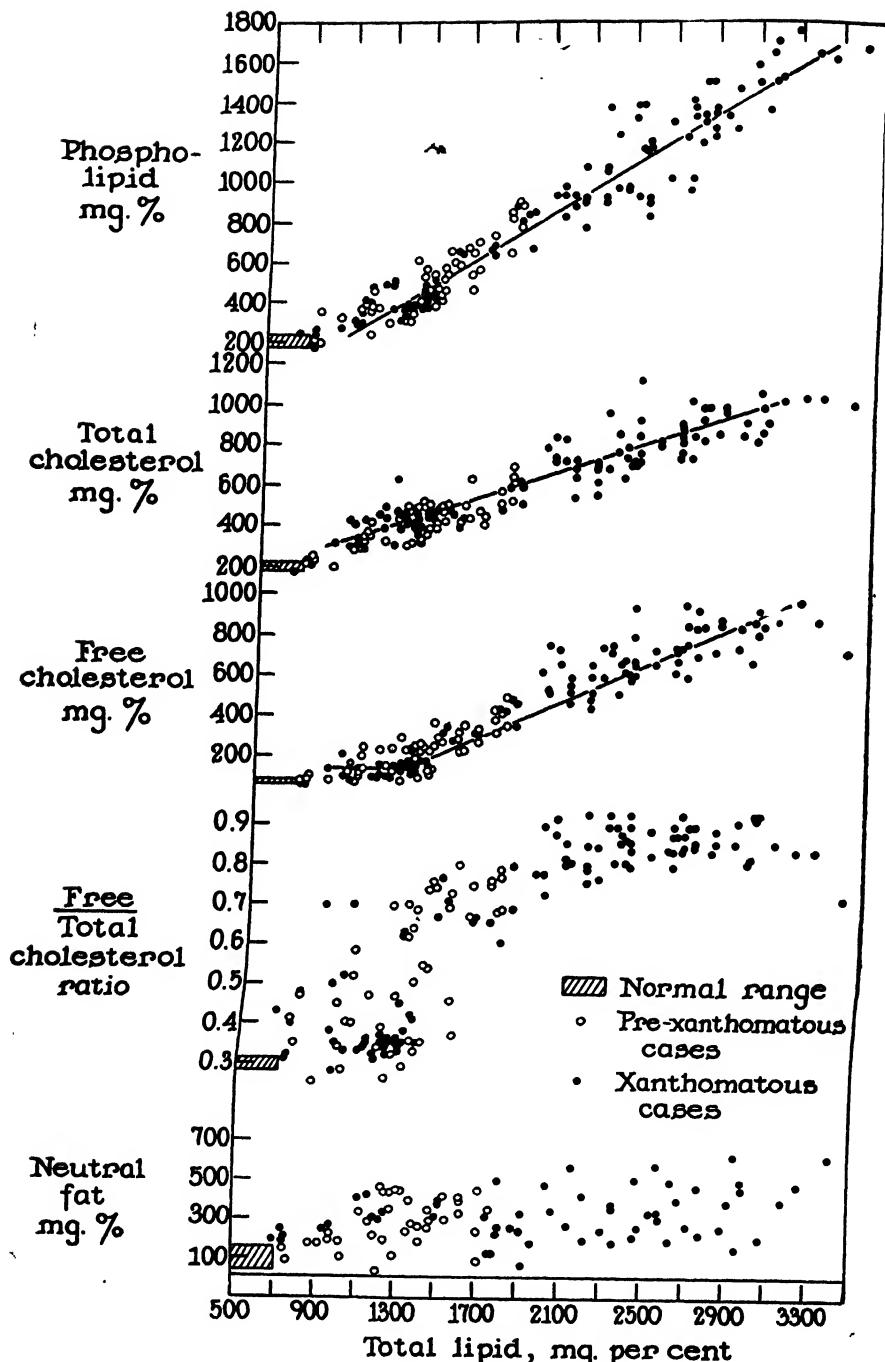
and who show the least elevation of total lipids. Two of the four patients whose xanthomata regressed had parallel decreases in bilirubin and lipid levels. However, in a third, where the decrease in serum lipids coincided with disappearance of xanthomata in the terminal year, there was a steady rise in bilirubin levels. Independent variation of serum bilirubin, serum lipids, and alkaline phosphatase activity has been noted repeatedly in this group, although, as Figure 4 shows, there is a general tendency for correlation in the group as a whole.
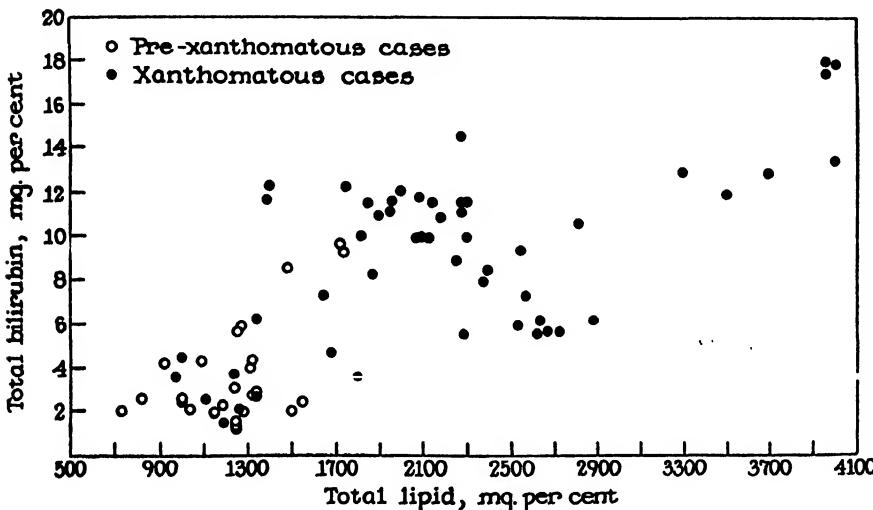
FIG. 4. Correlation between total serum lipid and total serum bilirubin concentrations repeatedly determined in 18 patients with primary biliary cirrhosis. Symbols as in Figure 3.

Laboratory evidence of the degree of biliary obstruction in these patients, when sought at monthly or more frequent intervals, has shown striking spontaneous variability. Complete obstruction has not been seen in a single patient in this series, as measured by urine and feces urobilinogen and urine bile, but when measured frequently over prolonged periods these indices of partial biliary obstruction have showed significant periodic rises and falls. Over longer intervals there have also been significant variations in serum bilirubin and serum lipid levels, as illustrated by the following two cases. In Figure 5 are

FIG. 3. Serum lipid fractions repeatedly determined in 18 patients with primary biliary cirrhosis, plotted against total lipid concentration for each serum, as compared to normal range. Xanthomatous cases (closed circles) = severe generalized flat or tuberous xanthomata. Pre-xanthomatous cases (open circles) = all others, including those with xanthelasma only.

charted the levels of total lipids and total bilirubin in a patient followed since 1943. This patient developed flat xanthomata of the hands in January, 1943, three months after the onset of jaundice and pruritus, and in \pri 1945, generalized tuberous xanthomata appeared which rapidly became mor ·xtensive and disabling than any encountered in this series of patients. Four major spontaneous rises and falls in lipid levels occurred between 1943 and 1948. In 1948, when for the first time total lipids returned to normal limits her xanthomata rapidly diminished in size and at present have almost completely
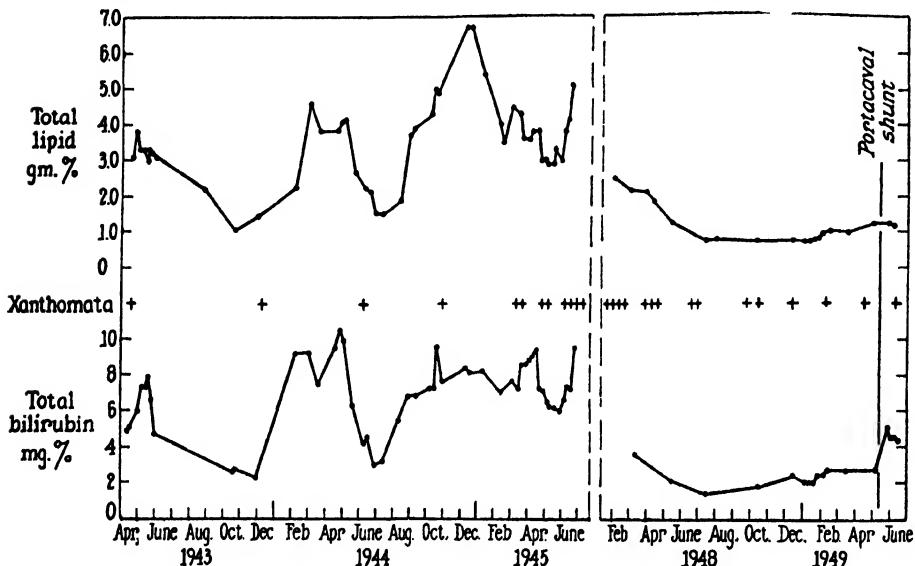
FIG. 5. Variations in total serum lipid and total serum bilirubin concentration in a 35 year old female with onset of primary biliary cirrhosis in October, 1942; resolution of xanthomata with fall in lipids. Portacaval shunt in May, 1949, for relief of portal hypertension and hemorrhage; at present in excellent general condition.

disappeared. The soybean lecithin administered by mouth during this last period of alleviation of biliary obstruction might have received credit for the improvement, had it not also been given to five other patients in this series *without effect. The changes in bilirubin and lipid levels in this patient are more strikingly parallel than have been observed in others in this series.*

Figure 6 illustrates the appearance of generalized tuberous and flat xanthomata in a patient two years after the onset of her illness. When first seen in January, 1948, she was found to have a pinpoint yellow deposit in one eyelid. On the basis of the finding of total serum lipids of more than 2000 mg.%, it was predicted that this patient would progress from her current pre-xanthomatous condition to that of florid xanthomatosis. This occurred three months
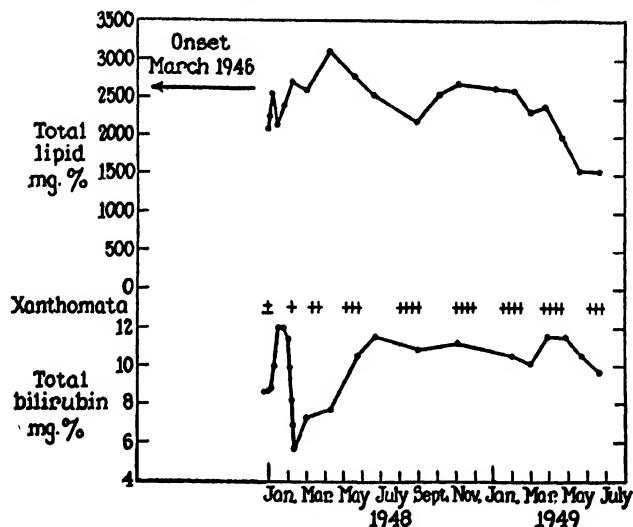
FIG. 6. Predicted appearance of severe generalized xanthomata in a 48 year old female with primary biliary cirrhosis for two years. In April, 1949, a fall in total lipids preceded a beginning resolution of xanthomata.
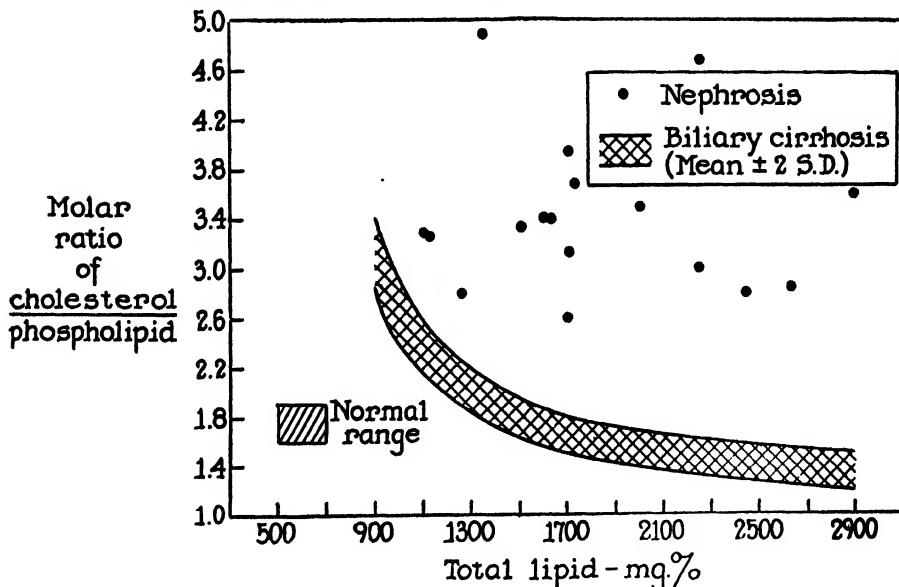
FIG. 7. Range of molar ratios of free plus esterified cholesterol to total phospholipid in normal sera and in 95% of data of Fig. 3, plotted against total serum lipid concentration. Nephrotic sera plotted individually. (Total phospholipid calculated as lecithin, and esterified cholesterol as cholesterol palmitate.)

later. Coincident with the administration of desoxycholic acid oral¹, (3 gm. per day) she experienced marked relief from abdominal bloating nausea, anorexia and bowel frequency, and shortly thereafter showed a dramatic fall in total serum lipids. She now shows softening and decrease in size of her xanthomata. Figure 6 also illustrates a dramatic change in bilirubin levels produced by nitrogen mustard in January, 1948, with red blood cell destruction and rise in bilirubin followed by anemia and secondary fall in bilirubin. Despite these variations there was no essential change in her intrahepatic "obstruction," and no significant change in serum lipids. By October, 1949, her total lipids had fallen to 600 mg.%, serum bilirubin to 4 mg.%, and her xanthomata continued to show dramatic resolution.

The possibility of a relationship between the ratio of hydrophobic/hydrophilic serum lipids and atheromatosis has been suggested (14, 15). In Figure 7 the molar ratio of cholesterol/phospholipid at various levels of total lipid has been calculated from the 150 lipid patterns of Figure 3 within limits including 95% of the data. This range is compared with individual determinations in patients with the nephrotic syndrome. In biliary cirrhosis it is seen that the higher the total lipid, the lower the cholesterol/phospholipid ratio, while in nephrosis this ratio is increased over normal at all total lipid levels.

DISCUSSION

Only seven of the 18 patients in this series showed the full-blown picture of xanthomatous biliary cirrhosis (5). The other 11 patients showed various stages in the development of this picture, some with xanthelasma alone and others with no xanthomata. Determination of total lipid levels at frequent intervals permitted the prediction of generalized xanthomatosis in two patients and the resolution of xanthomata in four. Thus, a definite pre-xanthomatous stage in this disease can be characterized, and the transition into and out of the full-blown xanthomatous stage is seen to depend upon the degree of elevation of the serum lipid level. Patients whose lipid levels never exceed 1800 mg.% remain arrested in the pre-xanthomatous phase of the disease. These patients are more numerous than those with the full-blown disease.

The characteristic serum lipid picture in this group of patients affords an objective measure by which the efficacy of various therapeutic agents may be judged. The use of a rapid turbidimetric method (10, 11) for determination of total serum lipid concentration has been of great assistance in this regard. Agents which have been administered in this series without significant alterations in lipid or bilirubin levels in the serum include: cholesterol-free diet, low fat diet, high protein diet, thyroid, soybean lecithin, intravenous serum albumin, tocopherol, nitrogen mustard, intravenous and intramuscular liver extract, and lipocaic. Desoxycholic acid appeared to have a lowering effect on the serum lipids in two of the patients. A full diet including fat with supplemental doses of fat-soluble vitamins was found necessary for adequate suppor-

tive treatment. The marked spontaneous variation in lipid and bilirubin levels which has been demonstrated in these patients necessitates prolonged observation for proper evaluation of therapeutic measures. Evaluation of therapy also is complicated by the fact that late in the disease, along with the development of signs of portal hypertension, there is a pronounced fall in serum lipid levels which may be accompanied by decreases in bilirubin and alkaline phosphatase activity, along with beginning resolution of xanthomata. This natural course of the disease must be differentiated from therapeutic success. Lacking knowledge of the exact means by which biliary obstruction (be it functional or mechanical) leads to elevation of serum lipids, it is evident that the mechanism by which the amelioration of obstruction is followed by a fall in lipids and disappearance of xanthomata also remains entirely conjectural.

The serum lipid values reported by MacMahon and Thannhauser (5) in their six patients with "xanthomatous biliary cirrhosis" differ in two respects from the values given in the present report. Three of their cases showed major elevations of cholesterol esters, whereas in the present series there has been a consistently major elevation of free cholesterol. Since the Schoenheimer-Sperry (9) method has been used in both laboratories, this discrepancy is difficult to explain. Secondly, although MacMahon and Thannhauser report extremely low figures for neutral fats, recalculation of their data by the formula of Thannhauser and Reinstein (16) shows significant elevations of neutral fats in some cases. In the present series manometric estimation of total lipid carbon gives clearcut evidence of increased amounts of neutral fats in the sera of xanthomatous and pre-xanthomatous patients.

The importance of serum phospholipids as stabilizers of the serum lipid emulsion has recently been stressed in a study (14) in which the particle size of lipid droplets in serum has been related to the ratio between hydrophilic and hydrophobic lipid concentrations. The implications of this thesis for studies on the pathogenesis of atheromatosis have received support in the recent work of Ladd, Kellner and Correll (15, 17) which demonstrated a reduced incidence of experimental atherosclerosis in rabbits in the presence of a reduced ratio of cholesterol to phospholipid in the serum. The relatively normal cholesterol/phospholipid ratios in the present patients despite total lipids greater than 1800 mg.% is thus of interest, since it has been shown that with these greatly elevated total lipid levels skin xanthomata occur regularly. In contrast to the well-known incidence of premature atherosclerosis in nephrosis, in the present series of patients with biliary cirrhosis and skin xanthomatosis there has been no clinical, electrocardiographic or radiographic evidence of atherosclerosis or coronary disease, and in four autopsied cases the degree of atherosclerosis was entirely commensurate with the age of the patient. The factors leading to xanthomatosis of the skin appear, therefore, to differ strikingly from those which determine the presence of arterial atheromatosis.

The serum lipid pattern which is characteristic of "primary biliary cirrhosis"

has also been found in this Hospital in obstructive jaundice of other types: in extrahepatic biliary obstruction (two cases), occasionally in congenital bile duct atresia (two cases), and in severely jaundiced patients with infectious hepatitis (five cases), and has elsewhere been reported in these conditions (18–21) and in arsenical liver injury (22, 23). Moreover, it has been noted in experimental bile duct ligation in rats (24, 25). Thus, the occurrence of elevated serum lipids in a clear serum, with predominant proportions of phospholipids, free cholesterol, and neutral fat, in decreasing order, appears to be pathognomonic of chronic partial biliary obstruction, either intra- or extra-hepatic in origin. If serum lipids remain sufficiently elevated for a sufficient length of time, skin xanthomata can be predicted, and, if for any reason the obstruction is relieved, it can be expected that the serum lipids will fall to normal levels and xanthomata will resolve.

CONCLUSIONS

1. In 18 patients with primary biliary cirrhosis, the occurrence of skin xanthomata has been directly related to the degree of elevation of the total serum lipids.

2. All patients with prolonged elevation of total serum lipids above 2000 mg.% have developed severe generalized skin xanthomata. Patients with total lipids below 1300 mg.% have shown no xanthomata, while in the intermediate range xanthelasma has occurred.

3. The typical lipid pattern of this disease, both in its pre-xanthomatous and its xanthomatous phases, is characterized by significant elevations of phospholipids (lecithin), free cholesterol, and neutral fat, in order of decreasing magnitude, with clear non-lipemic serum.

4. Disappearance of skin xanthomata coincident with fall in total serum lipids is described for the first time, and occurred in four out of seven patients with severe xanthomata.

5. The development of skin xanthomata without significant arterial atheromata in these patients is discussed.

Acknowledgment

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THE RELATIONSHIP BETWEEN SERUM LIPIDS AND THE ELEC-TROPHORETIC PATTERN, WITH PARTICULAR REFERENCE TO PATIENTS WITH PRIMARY BILIARY CIRRHOSIS

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One of the major characteristics of the serum of patients with chronic biliary obstruction is a marked elevation in the serum lipids. This elevation is primarily due to an increase in the phospholipid fraction although the cholesterol and neutral fat are also high (1). The phospholipid has been shown to act as a natural emulsifying agent to hold the other lipids in a clear solution (2). The presence of clear serum along with marked abnormalities in total lipid made the sera from these patients an ideal source material for studying the relationship of lipid changes to alterations in electrophoretic patterns. Analyses of these srea indicated a correlation between the total lipid level and the area of the beta globulin peaks determined electrophoretically.

Materials and Methods

Sera were obtained from 11 patients in the fasting state and were subjected to electrophoretic analysis. Patients Nos. 1 to 8 suffered from unexplained biliary cirrhosis as defined in the accompanying report (1). Patient No. 9 was a normal young adult. Patients Nos. 10 and 11 had severe post-necrotic cirrhosis proven by autopsy. Their sera were included in this study for contrast because of a marked reduction in total lipid. Table I lists the 11 patients according to the total lipid concentration in the serum.

Electrophoretic analyses were carried out by the method of Longsworth (3). The sera were dialyzed against barbital buffer (pH, 8.6; u, 0.1). Mobilities were calculated from the formula $U = \dfrac{hk_pA}{It}$ (4). Ascending patterns were used for the calculations of the beta globulin areas because of the interference in the descending patterns by the beta globulin disturbance. Serum was used in order to avoid the fibrinogen peak. Calculations of the areas in the electrophoretic patterns were expressed as per cent of the total area. This leads to some error in estimating beta globulin because of variations in albumin and gamma globulin. However, in each of the sera used the rise in the gamma globulin was approximately equal to the fall in albumin, thus minimizing the error in beta globulin variations. The observations of Armstrong, Budka, Morrison and Hasson (5) on the refractive index increments of various fractions of serum prepared by alcoholic fractionation furnish information that is helpful in calculating the amount of beta globulin directly from the electrophoretic pattern. Such estimations were attempted, utilizing the factor 1.71×10^{-4}. However, because of the unknown composition of the beta globulin peaks in these abnormal sera, the beta globulin was

expressed simply in terms of absolute units of area. This could be used only for com-
paring the different sera but served as a check of the validity of expressing the area
in terms of percentage of the total. Total protein nitrogen was determined for all of
the sera by micro-Kjeldahl analysis.

In experiments on the effect of removal of lipids, serum was diluted six times with
a solution containing 1% liquid phenol and 12% NaCl (6). This was not an extraction
procedure but simply one which appeared to interfere with the stabilizing effect of
lecithin on the serum lipids. Similar effects were obtained with the enzyme lecithinase.
The mixture was allowed to stand for 24 hours at 0°. C. The lipid was then centrifuged
into a pellicle at the surface at 10,000 r.p.m. Not all sera permitted the formation of a
pellicle with a clear subnatant solution. These were discarded. The pellicle was washed
and extracted with Bloor's alcohol-ether mixture. Cholesterol, phospholipid and total
lipid were determined on this extract. Nitrogen analysis was carried out on the ma-
terial remaining after alcohol-ether extraction. Electrophoretic patterns were made
on the subnatant solution after bringing the material to the original volume of the
serum in a dialysis bag in front of an electric fan and dialyzing against the standard
buffer solution.

Total lipid, cholesterol, and phospholipid determinations were obtained on the
same sera that were studied electrophoretically. The methods are described sep-
arately (1).

RESULTS

The characteristic feature of the electrophoretic pattern in the high-lipid
sera of the patients with biliary cirrhosis was a rise in the beta globulin com-
ponent. In addition, albumin was slightly reduced and the gamma globulin
was elevated. Figure 1 illustrates the ascending and descending patterns from
the sera of two of the patients with high total lipid concentrations. Patient
No. 2, with a total lipid of 2489 mg.% in the serum, showed an extremely large
area representing $beta_1$ and $beta_2$ globulins. Patient No. 7, with a somewhat
lower lipid level, also showed a very large beta globulin area but considerably
smaller than the combined $beta_1$ and $beta_2$ of patient No. 2. Figure 3 illustrates
the pattern of the serum from two other patients with total lipid concentrations
above 2000 mg.%.

In each case the area represented by the beta globulin peaks was proportional
to the total lipid concentration of the serum. Figure 2 illustrates the direct
relationship between these two variables in the serum of the 11 patients
studied. The beta globulin percentages in this figure represent the sum of the
various beta globulin peaks. A line drawn through the points extrapolates to
zero. For every 1000 mg.% rise in total lipid, there was approximately a 20%
increase in beta globulin area. Expression of the beta globulin in absolute units
of area instead of percentage of the total showed a similar straight line relation-
ship when plotted against total lipid concentration. This line also passed
through the zero point.

Table I contains the values for various lipid fractions along with the electrophoretic components for all of the sera covering a broad range of lipid concentration. In addition to the expression of the electrophoretic fractions as percentages of the total, the sum of the beta globulin components for each of the sera is presented in absolute terms as a multiple of the beta globulin
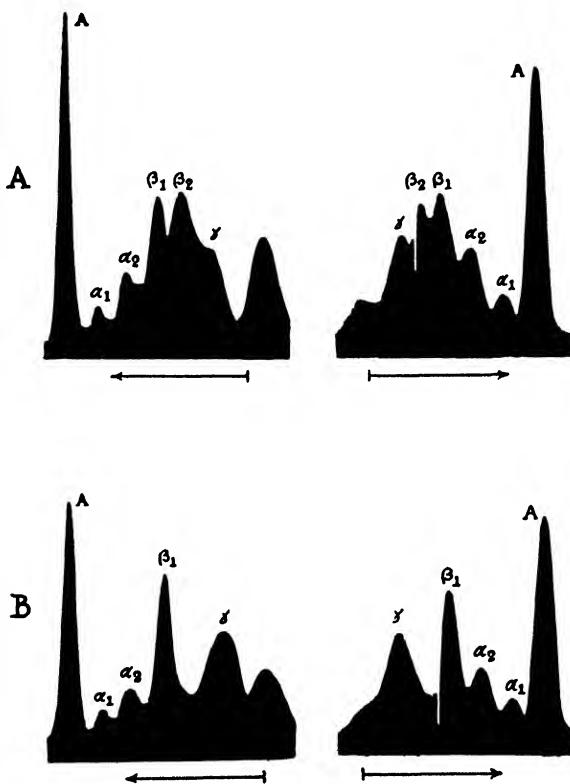
FIG. 1. Ascending and descending electrophoretic patterns from two of the patients showing elevated serum lipids. A—patient No. 2; total lipid, 2489 mg.%. B—patient No. 7; total lipid, 1317 mg.%. The increase in beta globulin is readily apparent.

area of normal serum. The predominant abnormality in the lipid pattern was and increase in phospholipid, although the free cholesterol and neutral fat were also increased. It was not possible to determine whether any one lipid fraction influenced the beta globulin area more than the others. The alpha globulins did not show an increase in the high-lipid sera similar to that observed for the beta globulins. However, if the alpha globulins were determined on the basis of the actual area present rather than as a percentage of the total, it was found that a definite rise did occur in the high-lipid ser~

The very large alpha₂ peaks reported by others (7) in nephrosis were not observed.

In the four sera with a total lipid concentration above 2000 mg.%, three showed a double beta globulin peak. The lipid composition of these four sera was very similar, as was the composition of the proteins. The reason for the single peak in the one case and the double peaks in the other three was not apparent. Table II shows the results of determinations of the mobility of the various beta globulin components. The component designated beta₁ had

TABLE I

The Lipid and Electrophoretic Composition of the Sera of the 11 Patients Studied

| Patient No. | Disease | Lipid mg. % | | | | | Electrophoretic area % of total | | | | | | $\frac{\beta\ globulin\ area}{Normal\ \beta\ globulin\ area}$[†] |
		Total lipid	Phospholipid	Neutral fat	Total choles- terol	Free cholesterol	Alb.	α_1	α_2	β_1	β_2	γ	
1	Biliary cirrhosis	2650	1300	348	950	864	29	2	5	22	26	16	6.0
2	Biliary cirrhosis	2489	1047	527	853	750	26	4	11	16	25	18	5.1
3	Biliary cirrhosis	2400	1220	293	810	682	27	3	9	20	28	13	5.8
4	Biliary cirrhosis	2200	980	451	732	671	26	5	9	43		17	4.9
5	Biliary cirrhosis	1560	620	327	453	186	35	6	11	21*		27	2.5
6	Biliary cirrhosis	1340	594	216	417	229	36	3	10	27		24	2.3
7	Biliary cirrhosis	1317	553	123	492	244	28	4	9	29		30	2.6
8	Biliary cirrhosis	811	310	116	281	107	47	5	10	17		21	1.7
9	Normal	584	183	124	191	48	63	4	8	13		12	1.0
10	Post-necrotic cirrhosis	323	110	66	103	30	43	6	12	7		32	0.5
11	Post-necrotic cirrhosis	296	120	91	72	51	41	5	7	8		39	0.5

* β_1 for this serum represents the sum of β'_1 and β_1.

† The absolute β globulin area of normal serum No. 9 was used for this comparison.

the mobility of the beta globulin seen in normal serum. The concentration of this component was increased in all of the high-lipid sera. The component beta₂ showed a much slower mobility and was seen only in the very high-lipid sera. Another component, beta prime₁, with unusually rapid mobility was observed in only one of the sera (patient No. 5).

In order to determine the effect of lipid removal on the electrophoretic pattern of the high-lipid sera, electrophoretic patterns were carried out before and after diluting serum with a high salt 1% phenol solution. This procedure has been demonstrated to cause 85 to 95% of the lipid of the serum to separate from the solution along with approximately 5% of the protein (6). Figure 3 illustrates these patterns from two of the sera. The procedure did not alter significantly the albumin and gamma globulin components. However, the
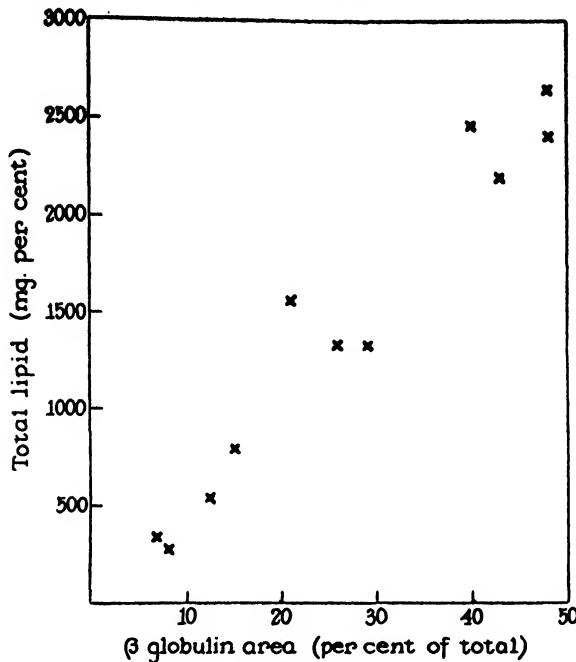
FIG. 2. The relationship between the total lipid concentration and the electrophoretically determined beta globulin area for the sera of the 11 patients studied.

TABLE II

Mobilities of the Various β Globulin Peaks Encountered in the Sera of the 11 Patients Studied

Patient No.	Mobilities × 10⁵		
	β'_1	β_1	β_2
1		2.73	1.93
2		2.90	2.00
3		2.86	1.97
4		2.90	
5	3.2	2.80	
6		3.05	
7		2.84	
8		2.80	
9		3.10	
10		2.80	
11		2.95	

beta globulin area was reduced markedly. In Figure 3A the total protein nitrogen of the serum was 1.07%; 0.06 gm.% protein nitrogen was removed on centrifugation. The total lipid of this serum was 2200 mg.%; 2070 mg.%

were removed on centrifugation. The electrophoretic area lost by the procedure represented 33% of the original total area. The nitrogen-lipid ratio for the area lost could be calculated readily. The factor that should be used for the conversion of nitrogen to lipoprotein for the area lost is not clear because it probably represents various lipoproteins. If the ordinary protein factor 6.25 is used, the area lost represents 17% protein. Similarly, for Figure 3B, the area lost represents 14% protein. This would indicate a lipid content of 83% and 86% for the areas lost.

Numerous other analyses of the amount of protein which separated with lipid by the high salt 1% phenol procedure indicated that the composition of

A   B
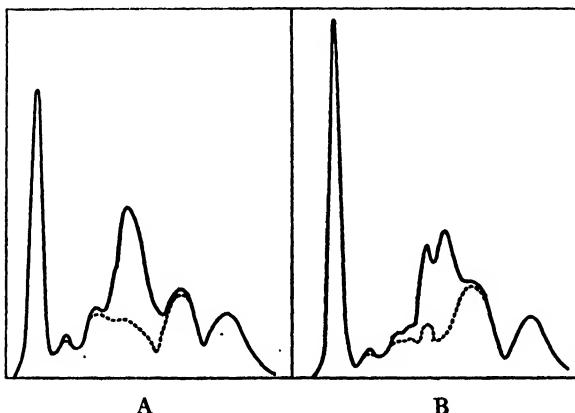
Fig. 3. Ascending electrophoretic patterns from two high-lipid sera before and after separation of 95% of the serum lipids by dilute phenol solution. Dotted lines represent the areas obtained by the procedure.

the separated material averaged 14% protein and 86% lipid. These observations have been published separately (6). Sera from normal individuals showed a higher protein content of the separated material. The average value obtained was 28% protein. These results suggest that the elevation in total lipid in the patients under discussion in this study was not associated with a proportional increase in tightly bound lipid-protein complexes.

DISCUSSION

The electrophoretic determinations on the sera of the 11 patients included in this study indicate that the beta globulin area is the primary component which is increased with increasing lipid concentrations. The alpha globulin showed minor changes. The direct correlation of total lipid and total beta globulin area was striking and suggests that the concentration of beta globulins and lipoproteins in serum is determined by the total lipid level.

Just how the lipoproteins which make up the beta globulin area in the high-lipid sera differ from those normally present in serum is not entirely clear. Two differences, however, have been noted. First, the amount of protein tightly bound to lipid was not elevated to the degree expected from the total lipid increase. Secondly, a slowly moving component, beta₂, appeared in the electrophoretic patterns of three of four sera containing more than 2000 mg.% total lipid. This component contained a large part of the total lipid of the serum. The possibility is raised that another protein takes over the function of combining with the large excess of lipid in these sera, particularly since the normal beta₁ component is markedly increased. Whether this represents a new protein or increased amounts of a protein present in normal serum is not clear.

The slowly moving beta₂ component showed approximately the same mobility as fibrinogen. The fact that this peak did not represent fibrinogen was verified by determination of fibrinogen levels in the plasma of these patients. Although a slight elevation was found, this was not nearly sufficient to cause the marked beta₂ peaks. In addition, since serum was used for the electrophoretic analyses, the major portion of the fibrinogen had been removed.

In the presence of high-lipid concentration the error involved in the usual calculation of various protein fractions from electrophoretic patterns as percentage of the total area is apparent from these studies. In the one illustration shown, 48% of the total electrophoretic area represented beta globulin but this contained only a small percentage of the total protein nitrogen. Similar conclusions have been reached by other observers who extracted the lipids of serum with ether or acetone in the cold (7, 8). Accurate estimation of protein concentration in high-lipid sera such as those from patients with biliary cirrhosis and nephrosis can only be obtained by direct calculation from the refractive index increment of the proteins.

SUMMARY

1. Electrophoretic patterns of the sera of patients with unexplained biliary cirrhosis containing high-lipid concentration showed marked increases in the beta globulin components.

2. The total beta globulin area was directly proportional to the total lipid concentration of the serum. Determinations were carried out over a total lipid range from 300 to 2700 mg.%.

3. The high-lipid sera all showed a beta globulin component with a mobility close to that of the beta globulin seen in normal serum. An additional component with a slower mobility appeared in three of the four sera with very high lipid concentration.

4. Attempts were made to determine the composition of the beta globulins by means of electrophoretic patterns before and after separation of the lipids from serum.

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THE IMMUNOLOGICAL DETERMINATION OF HUMAN ALBUMIN IN BIOLOGICAL FLUIDS

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The determination of serum albumin by an immunological method was first described by Goettsch and Kendall (1) in 1935 in dogs. Subsequently, the technique was applied to human serum (2–4). These early applications of the immunological technique were hampered by the difficulty in obtaining pure samples of human albumin for the preparation of antisera. Recent advances in the fractional precipitation of plasma proteins with alcohol have made large amounts of albumin available of sufficient purity to permit its use as an antigen. Such material was used by Chow (5) in the estimation of serum albumin. He showed good agreement with simultaneous electrophoretic determinations. Kabat, Glusman, and Knaub (6) have prepared antiserum to γ-globulin as well as to albumin from material obtained by alcohol fractionation, which proved useful in estimating these proteins in biological fluids.

In the present paper further evidence is presented for a close relationship between albumin concentrations determined immunologically and electrophoretically in sera with extreme differences in protein fractions. The application of the technique to a variety of biological fluids is demonstrated. In addition, the use of a new method for the quantitative estimation of protein in antigen-antibody precipitates is described. The method employs the ninhydrin reaction in a procedure similar to that of Moore and Stein for amino acid analyses (7).

Materials

Albumin Standard—The Red Cross concentrated human serum albumin produced by E. R. Squibb and Sons was used. This usually contained between 24 and 25 gm. of albumin per 100 cc.

Rabbit Antiserum—The above albumin solution was used for injecting rabbits in a manner similar to that described by Chow (5). Equal volumes of albumin, 5 mg. per cc. or 1 mg. per cc., and 0.5 per cent alum were used. The antiserum obtained was centrifuged prior to use. This was important even if the antiserum appeared clear (4).

The reagents used for the ninhydrin procedure were the same as those described by Moore and Stein (7).

Procedure

The method is carried out by diluting serum or other fluids with saline to give a final albumin concentration between 0.01 and 0.03 mg. per cc. A 0.5 cc. aliquot is placed in a small cuvette (10 × 75 mm.). To the cuvette 0.2 cc. of antiserum is then added and the mixture is allowed to stand for 1 hour at room temperature. At the end of this time the precipitate is handled in a manner similar to that described by Kabat and Mayer (4). Following centrifugation and pouring off of the supernatant with drainage on filter paper, 2 cc. of saline are added to the packed precipitate. This is broken up by rotating and shaking the tube and is recentrifuged. The procedure is repeated two more times, making a total of three washings with saline. The washed precipitate is dissolved in 0.1 cc. of NaOH (0.1 N), and 0.5 cc. of ninhydrin solution is added. The tubes are then placed in a boiling water bath for 20 minutes, after which 2 cc. of 1:1 water-propanol diluent are added. Following shaking, the tubes are read in a Coleman junior spectrophotometer at 570 mμ. A saline-antiserum mixture, carried through the entire procedure, is used as a blank.

A standard curve is prepared by using solutions containing 0.01, 0.02, and 0.03 mg. per cc. of albumin in place of serum in the above procedure. These dilutions were made up from a stock solution, the albumin of which had been determined by micro-Kjeldahl analysis with the nitrogen factor 6.25. The color produced with serum may be transferred directly to mg. of albumin from this standard curve.

EXPERIMENTAL

In carrying out a large number of albumin determinations by the immunological technique, the importance of conserving antiserum soon became apparent. The method as described by Chow (5) requires the use of 2 cc. of rabbit serum for each determination and, if these are done in duplicate or triplicate, 4 to 6 cc. of rabbit serum are needed. The phenol reagent as employed by Heidelberger and MacPherson (8) and the protein absorption in the Beckman spectrophotometer by the procedure of Eisen (9) and Gitlin (10) are known to be more sensitive methods of analyzing quantitatively the protein in antigen-antibody precipitates. A comparison of these various techniques, together with other methods of protein estimation, was therefore undertaken. Table I illustrates the approximate minimal amount of protein (albumin) that could be measured by various procedures under comparable conditions. The amount of protein that gave a final optical density reading of 0.07 in a volume of 2.5 cc. in the spectrophotometer was used for comparison. The direct measurement of protein in the Beckman spectrophotometer was carried out at a wave-length of 287 mμ in a solution of 0.1 N NaOH. The smallest

amounts of protein were detected by the ninhydrin procedure of Moore and Stein (7).

This method also has the advantage that it has been critically worked out to give a stable color and highly reproducible results with amino acids. The method is not specific for amino groups in proteins. Color is also given by peptides, amino acids, amines, and NH_3. The presence in the precipitate of any of these substances except NH_3 is unlikely, and with minimal precautions contamination with NH_3 can be prevented.

Various proteins give somewhat different intensities of color with the ninhydrin reagent. Fig. 1 illustrates the comparison between albumin and an electrophoretically homogeneous γ-globulin preparation in respect to

TABLE I

Comparison of Sensitivity of Various Methods of Protein Estimation

The amount of protein giving a final optical density reading of 0.07 in the spectro-photometer is listed. The numbers in parentheses refer to the bibliography.

Method	Protein
	mg.
Ninhydrin (7)	0.02
Colorimetric Kjeldahl (12)	0.06
Biuret reagent (13)	0.80
Beckman spectrophotometer (10)	0.09
Phenol reagent (8)	0.10
Micro-Kjeldahl*	1.20

* The figure listed for the micro-Kjeldahl is the minimal amount that could be conveniently titrated.

intensity of color at various nitrogen concentrations as determined by micro-Kjeldahl analysis. It is apparent that albumin gives a more intense ninhydrin reaction. However, for the analysis of the immune precipitate this difference offered no interference because of the constant ratio of albumin to antibody.

Although it was demonstrated by Chow (5) that albumin could be determined quantitatively in the zone of antibody excess, further experiments were carried out to determine the exact point where an approximate linear relationship between albumin concentration and precipitated protein ended. Varying quantities of pure albumin and also serum were tested with a constant amount of antiserum. The immune precipitate was estimated with the ninhydrin reagent. Fig. 2 illustrates the results for pure albumin and also for serum. The albumin content of this serum had been determined immunologically. The amount of antiserum used for each determination in this experiment was 0.3 cc. The curves for
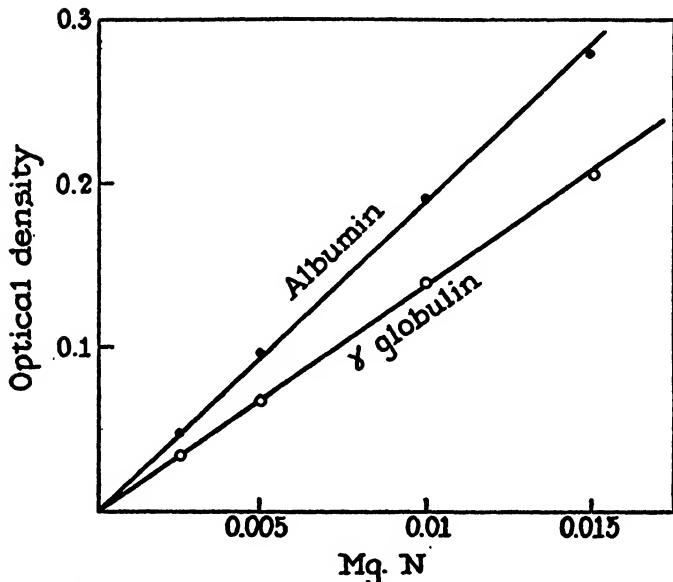
FIG. 1. Comparison of the intensity of color produced with ninhydrin by albumin and γ-globulin per unit of nitrogen.
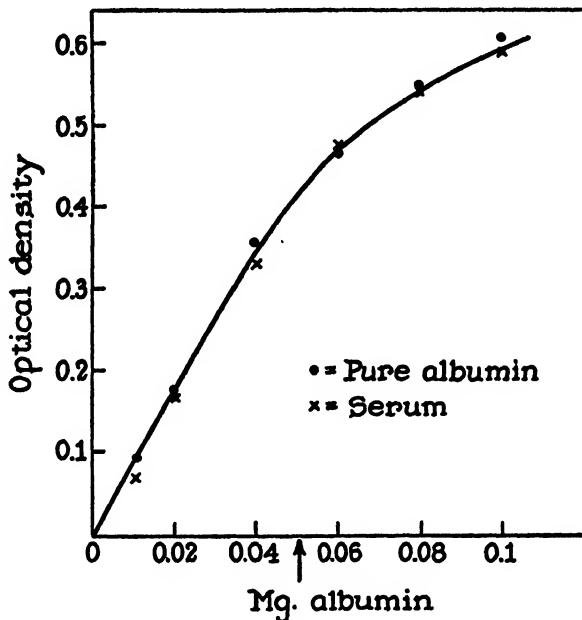
FIG. 2. Effect of increasing amounts of antigen (albumin and serum) on the quantity of antigen-antibody precipitate as measured with the ninhydrin reagent. The antibody concentration was kept constant.

the albumin and serum were completely parallel, leveling off above a concentration of 0.05 mg. of albumin in each case. There was no interference from the proteins other than albumin in the serum. The relationship between albumin concentration and immune precipitate approaches linearity below 0.05 mg. of albumin. Various batches of antiserum showed slightly different critical levels for the ratio of albumin to antiserum below which this relationship held. However, if the ratio of mg. of albumin to cc. of antiserum was kept below 0.15, accurate quantitative results for albumin could always be obtained. In the experiments described in this report the ratio was kept below 0.1.

TABLE II

Variability of Albumin Values Obtained in Two Sera and One Ascitic Fluid with Three Dilutions of Antigen, Analyses in Triplicate

Serum or ascitic fluid	Albumin			Average
	Experiment 1	Experiment 2	Experiment 3	
cc.	*gm. per cent*	*gm. per cent*	*gm. per cent*	
0.0006	0.612	0.595	0.597 ⎫	
0.0012	0.563	0.564	0.571 ⎬	0.578 ± 6%
0.0018	0.562	0.573	0.571 ⎭	
0.0002	3.10	2.90	2.98 ⎫	
0.0004	2.97	2.83	2.85 ⎬	2.92 ± 3%
0.0006	2.90	2.92	2.90 ⎭	
0.0002	4.32	4.23	4.25 ⎫	
0.0003	4.22	4.10	4.20 ⎬	4.21 ± 3%
0.0004	4.25	4.20	4.15 ⎭	

Table II shows the results of three series of analyses on the same specimens of serum and ascitic fluid. Each analysis was carried out at three dilutions of antigen, all of which were below the critical level of antibody excess. The reproducibility of the results was slightly better at a single concentration of antigen, but even with the use of various dilutions of antigen the values fell within approximately ±5 per cent of the average.

Incubation of the antigen-antibody mixture was not found to have any advantages over simple standing for 1 hour at room temperature. The correlation between various concentrations of serum and standard albumin solution was equally good at room temperature. In addition, standing at 0° for various periods after incubation did not offer any advantage. With antiserum which was not completely fresh, higher blanks were sometimes obtained after prolonged standing.

Electrophoretic Correlation—The immunological procedure for the determination of albumin was carried out on twenty-two sera and three
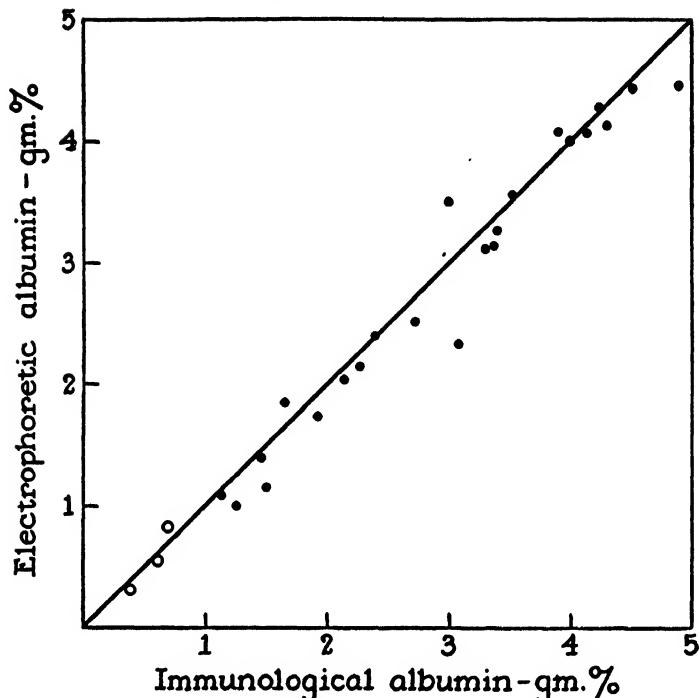
Fig. 3. Correlation between albumin concentrations determined immunologically and electrophoretically. O represents ascitic fluid.

TABLE III

Comparison of Electrophoretic and Immunological Determinations of Albumin in Sera of Widely Divergent Protein Composition

Total protein	Electrophoretic analysis		Immunological analysis
	γ-Globulin	Albumin	Albumin
gm. per cent	*gm. per cent*	*gm. per cent*	*gm. per cent*
2.3	0.3	1.1	1.2
4.3	0.6	2.1	2.3
7.6	1.0	4.4	4.9
9.2	6.1	2.5	2.8
11.4	7.1	3.1	3.6
12.1	7.2	3.6	3.4

ascitic fluids which previously had been subjected to electrophoretic analysis for other purposes.[1] Some of the sera had been stored at 0° for periods

[1] Sera with elevation in lipides were not used because of the increased error involved in correlating electrophoretic area with protein concentration.

of 6 to 18 months. No sera were used which showed evidence of protein sediment. Electrophoretic patterns were obtained by the method of Longsworth (11). The amount of albumin was calculated by multiplying the area of the albumin peak in per cent by the total protein as determined by micro-Kjeldahl analysis. The factor 6.25 was used for the calculation of protein from nitrogen, both for the electrophoretic albumin and for the standard curve of the immunological procedure. Fig. 3 illustrates the close correlation between the albumin determinations by the electrophoretic and immunological procedures. 75 per cent of the points fall below the line for perfect correlation, indicating slightly higher values for the immunological procedure. On averaging all the values, this difference amounts to 0.12 gm. per cent.

Table III lists the albumin determinations by the two methods in six sera of widely divergent protein composition. The concentration of γ-globulin is also listed, because this represented the chief abnormality in the high protein sera. The high γ-globulin sera were obtained from a group of patients with unusual liver disease. The correlation between the two albumin methods was similar, regardless of the concentration of other proteins.

DISCUSSION

The estimation of the quantity of antigen-antibody precipitate by the ninhydrin method of Moore and Stein (7) appeared to have certain advantages for measuring albumin over other methods now in use. Smaller quantities of antiserum were necessary, 0.2 cc. of antiserum or less depending on the potency of the antiserum. As little as 3 γ of albumin could be accurately measured in biological fluids. The method was more sensitive than that of Heidelberger and MacPherson (8) utilizing the phenol reagent and that of Gitlin (10) employing absorption in the Beckman spectrophotometer. The color produced with ninhydrin was more stable than that with the phenol reagent.

Measurements of albumin concentration were carried out in serum, ascitic fluid, chest fluid, urine, and tissue extracts. The method proved of particular value when the concentration of albumin was particularly low and when albumin determinations by the Howe method would have little significance. Electrophoretic determinations are also difficult under such conditions because of the necessity of concentrating the material prior to analysis. In urines, for example, this often means concentration of pigments which interfere with the determinations.

Comparison of albumin levels in sera of widely divergent protein concentrations by the immunological and the electrophoretic methods showed a good correlation. The correlation was equally good at the low albumin

levels existing in ascitic fluid. Electrophoretic determinations were not carried out on urines, but the results would be expected to be similar to those in ascitic fluids.

SUMMARY

The reaction of ninhydrin with NH_2 groups has been utilized as the basis for a photometric determination of protein in antigen-antibody precipitates. Comparison with common methods of protein determination indicated that this was more sensitive. The procedure permitted the use of smaller quantities of antiserum for the determination of albumin in biological materials. The correlation between the immunological and the electrophoretic methods of determining albumin was good regardless of the concentration of albumin and other proteins. Slightly higher values for albumin were obtained by the immunological procedure. The method proved particularly useful for determinations of small quantities of albumin in biological fluids other than serum.

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PRESSOR SUBSTANCES IN ARTERIAL HYPERTENSION

ACTIVITY AND AMINE CONTENT OF CRUDE EXTRACTS OF BLOOD*

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A search for pressor substances has been conducted for many years both in human beings and in experimental animals (1). Usually these experiments resulted in failure to demonstrate any positive effect. In dogs, however, Solandt, Nassim and Cowan (2) were able to demonstrate some effect by transfusing blood from hypertensive to nephrectomized animals. Freeman (3) reported that the hypertensive dog's blood differed from normal blood when transfused. In the blood of human beings with so-called 'essential hypertension,' it is undecided, however, whether or not vasoconstrictor substances exist, although the weight of evidence is in favor of their presence. And yet, Host (4), Pickering (5) and Prinzmetal and his colleagues (6) were unable to demonstrate any effect of hypertensive venous blood on the blood pressure of normal subjects in cross transfusion experiments, even if large quantities were used.

To know whether or not pressor substances are present in blood of cases of arterial hypertension in man is of the utmost importance. A series of experiments was begun (7), therefore, in an attempt to demonstrate their presence. In preparing extracts, the following assumptions were made: *a*) that arterial blood contains more of the hypothetical pressor substances than does venous blood. This assumption was based on the possibility that the kidneys or other organs with direct venous return to the heart might elaborate these substances and that they would be metabolized in part during passage of blood through the arterioles, just as happens in the case of epinephrine; *b*) that the substances are present in very small amounts; *c*) that they are unstable, being easily oxidized or inactivated perhaps by enzymes in the blood; *d*) that possibly they are more or less complex amines.

This paper reports the progress made in this search prior to interruption by the war. Evidence is presented on the presence of pressor substances. It

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appears that, when acidified, these are soluble in 90 per cent alcohol and insoluble in petroleum ether and in ether.

Methods

Preparation of Blood Fractions. Arterial blood was drawn directly into an Erlenmeyer flask containing an amount of 95 per cent alcohol equal to 2 to 3 times the volume of blood (i.e. 200 cc. of blood to 400–600 cc. of alcohol)

Preparation of Blood Extracts

Step 1 One volume of blood precipitated with 10 volumes of 95% alcohol

Step 2   Filtered

     Filtrate acidified with HCl   Precipitate discarded

Step 3· Concentrated to dryness in vacuo

Step 4 Extract with 90% alcohol and centrifuge

     Supernatant   Precipitate discarded or dissolved in saline and tested as H fraction

Step 5 Freed of alcohol in vacuo and extracted with petroleum ether

Step 6 Water soluble extract freed of petroleum ether and centrifuged   Petroleum ether extract-add saline and distill with petroleum ether

     Saline solution, P fraction   Precipitate discarded

     Supernatant brought to 1/20 original volume of blood. A fraction   Precipitate discarded

FIG. 1. Preparation of blood extracts. The pressor activity of the various fractions of hypertensive blood in rats was as follows: *H* fraction, 1 of 4 injections; *P* fraction, 7 of 17, with 3 giving only immediate pressor effects; *A* fraction, 29 of 41, with 4 giving immediate responses (see text). Extracts of normotensive blood were active as follows: *P* fraction, 2 of 9 injections; *A* fraction, 4 of 25, with one immediate response.

(fig. 1). The amount of alcohol was small because the amount of blood to be drawn was not known. Later, alcohol was added to give a total of 10 volumes of alcohol. The precipitate was removed by filtration. Concentrated HCl was added to the filtrate (one cc. for each 200 cc. of blood). The filtrate was concentrated (usually overnight) to dryness *in vacuo* at 30 to 35°C. The dry residue was then extracted briefly several times with a minimum amount of alcohol, a volume approximately equal to the original amount of blood. It was centrifuged and the precipitate discarded. The supernatant fluid was freed of alcohol by vacuum distillation and the residue was extracted with petroleum ether.

The petroleum ether extract was discarded after preliminary tests with

saline extracts of it showed that little or no pressor material was present. The petroleum ether was removed by vacuum distillation. The residual aqueous layer was centrifuged to separate any insoluble material. If the volume of the aqueous residue was small, saline was added before centrifugation to $\frac{1}{20}$ the volume of the original blood. This preparation was labeled the A fraction. Other fractions are shown in figure 2 and in some instances are discussed later in the text. Before injection into rats to test the presence of pressor activity, the pH of the extracts was adjusted to approximately 7.4 with sodium bicarbonate or sodium hydroxide solution.

Measurement of Amines. For the purpose of estimating the relative concentrations of amines in the preparations from blood, a modification of Richter's method (8) was employed. To one cc. of material, 4 cc. of 2 M NaOH and 6 cc. of toluene were added. The mixture was shaken well for 5 minutes and centrifuged whenever necessary to separate the 2 layers. After the extraction, 4 cc. of the toluene extract were added to 4 cc. of an 0.08 per cent solution of picric acid in chloroform in a dry tube. The intensity of the yellow color formed was estimated in terms of the color developed by various amounts of isoamylamine extracted in the same manner. It was recognized that the procedure would not give precise information on the amine content because *1*) not all amines are extracted (for example, the hydroxy phenylamines), *2*) the simple amines do not give a color, and *3*) the various amine picrates have different transmission spectra. In some instances the amines formed picrates that crystallized. These were collected and tested for their pressor activity.

Tests for Pressor Activity. Preparations were tested in rats for their pressor activity. Rats weighing 200 to 350 gm. were anesthetized with sodium pentobarbital given intraperitoneally. Tracheotomy was performed because, when this anesthetic was used, rats often died of upper respiratory obstruction. The femoral vein was isolated and a blunt needle tied into it. The femoral artery on the opposite side was isolated. A few crystals of novocaine were placed on it followed by 2 or 3 drops of physiological saline solution to prevent spasm of the femoral artery, so as to permit the insertion of a 22- or 23-gauge needle connected with a Hamilton manometer. An adequate amount of heparin was then injected intravenously. Approximately 160 rats were used. One cc. of the extract, which corresponded to 20 cc. of original blood, brought to a pH of 7.4, was injected. The effects on the blood pressure were recorded on a photokymograph. An elevation of the diastolic pressure of more than 12 mm. Hg, 5 to 20 minutes after injection, was considered to be significant.

Sixty-five samples of human arterial blood taken from 23 patients suffering from arterial hypertension and from 22 normal subjects were extracted. Blood from 2 normal dogs and from one made hypertensive by wrapping the kidneys in cellophane was also tested. The amounts of the samples were usually 200 to 500 cc. Smaller amounts were also taken for special studies.

RESULTS

Types of Response. Certain preparations exerted a pressor effect in rats. Two types of response were noted. One resembled that seen after injections of familiar pressor substances, in that it occurred immediately, was over in from 2 to 5 minutes and was associated with widening of the pulse pressure. This was characteristic of several groups of extracts.

Modifications of A Fraction
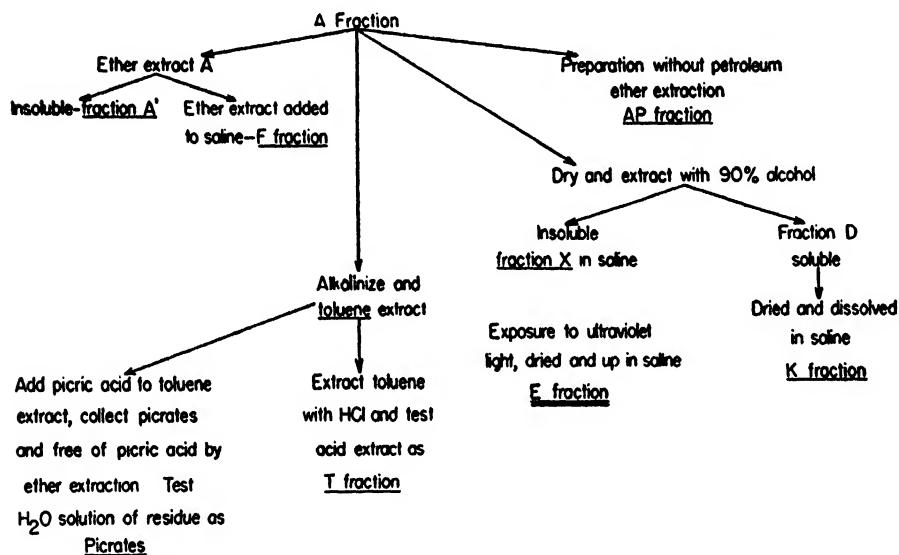
FIG. 2. Modifications of a fraction gave positive prolonged pressor effects in rats as follows: Hypertensive, A', 2 of 6 injections; F, none of 5; AP, 3 of 8; X, 2 of 7; K, 2 of 5; E, 3 of 18, with 8 others giving immediate pressor responses; T, 2 of 4; and Picrates, 9 of 13, with one immediate response. Normotensive, X, none of one injection; E, 3 of 10; T, 1 of 3; and Picrates, 1 of 5.

The second type, usually preceded by a depressor effect, became obvious 2 to 5 minutes after injection. It frequently lasted 20 minutes or longer (fig. 3). This response differed from that seen after injections of renin, angiotonin, epinephrine, tryptamine or isoamylamine, in that it was more prolonged. But it did resemble slightly the effect which follows the injection of tyramine, and even more that produced by phenylethylamine. Mixtures of active extracts of blood incubated with preparations of amine oxidase were inactive.

The effect of injections of extracts from hypertensive and from normal individuals differed. Pressor effects (in rats) were caused by a majority of
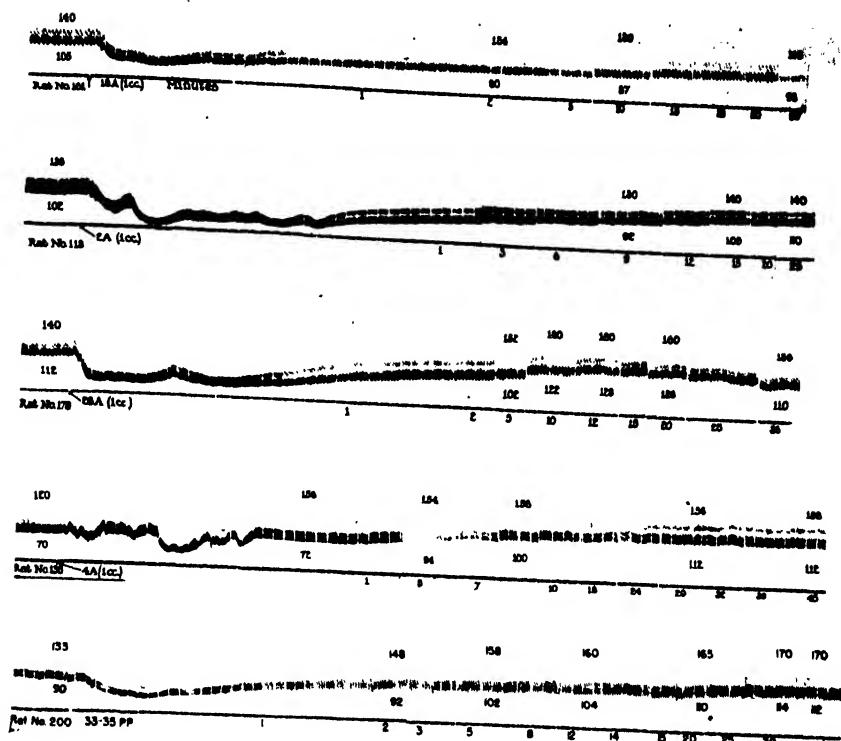
FIG. 3. Typical responses of rats' blood pressure to injections of extracts of blood. The blood pressure is indicated by the curve photographed from the Hamilton manometer. The figures above refer to the systolic, below to the diastolic pressure in mm. Hg. The time is recorded on the lower line at 2-second intervals, the numbers indicating minutes after injection. *Rat 161* was injected with extract 18A from a normal subject. There was an initial depression (considered + +) not followed by a rise. *Rat 118* was similarly injected with extract 2A from a patient exhibiting congestive heart failure. The initial depression (considered + + + +) was not followed by a significant rise. *Rat 178* was injected with extract 28A from a patient with arterial hypertension. The initial depression (+ + +) was followed by a typical delayed pressor response reaching its height of 160/128, an elevation of 20 16, in 12 minutes and gradually returning to normal levels after 25 minutes. *Rat 135* was similarly given extract 4A from a hypertensive patient. The depressor response in this case was of short duration (+) and was followed by a rise in blood pressure which lasted 45 minutes or longer. Note the initial widening of the pulse pressure. *Rat 200* was injected with mixed picrates (one cc.) and showed an initial depression (considered +) followed by a significant rise lasting 30 minutes.

those from hypertensive patients, and by only a minority from normal individuals.

TABLE I

Prolonged Pressor Activity of Various Active and Partly Active Fractions, and Amine Content of Extracts of Arterial Blood (Fractions A, A', AP and Picrates)[1] from Hypertensive and Normotensive Subjects

Patient No.	Sex	No. blood samples	Extracts tested Pos.	Extracts tested Neg.	Amine content $\lambda/cc.$[2]	Remarks
Renal Hypertension—Severe						
7	♂	2	3	2[3]	>10	1 Post-operative
8	♀	1	2	0	3	
9	♂	1	2	0	3	
12	♂	2	4	0	0	
15	♀	2	1	2		Post-operative
17	♂	1	0	1		
18	♂	2	3	0	>10	
19	♀	1	1	1	1.5	
21	♂	1	2	1	9	
22	♀	1	2	0	>10	Post-operative
27	♂	1	1	1	>10	Heart failure
Renal Hypertension—Moderate						
4	♂	3	4	1[3]	3.5	1 Post-operative
13	♀	1	1	1	9	
28	♂	3	5	2	3.5	
29	♂	1	1	0	4.5	
31	♂	1	3	0	9	
32	♂	2	4	1	5	
Total.	17	26	39	11		
Per cent positive.	94		78			
Neurogenic Hypertension—Severe						
1	♀	2	1	3	>10	
5	♂	5	0	8	>10	
Neurogenic Hypertension—Moderate						
16	♂	1	1	1		
25	♂	2	4	2	>10	
26	♂	1	1	3	>10	
20	♂	1	0	2	3.5	Pheochromocytoma
Total.	6	12	7	19		
Per cent positive.	67		27			

<div align="center">TABLE I.—*Continued*</div>

Patient No.	Sex	No. blood samples	Extracts tested		Amine content γ/cc.[2]	Remarks
			Pos.	Neg.		

<div align="center">*Normotensive Patients*</div>

Patient No.	Sex	No. blood samples	Pos.	Neg.	Amine content γ/cc.[2]	Remarks
2	♂	1	0	2	1	R.H.D. Heart failure
3	♂	2	0	3	1	A.H.D. Heart failure
6	♂	1	1	0	1	R.H.D. Heart failure
10	♀	1	0	1	0	A.H.D. Heart failure
11	♂	1	0	4	0	A.H.D. Heart failure
23	♀	1	0	1	1	Post-nephrectomy
34	♂	1	0	2	3.5	Glomerulonephritis
Totals..............	7	8	1	13		

<div align="center">*Normotensive Normal Subjects*</div>

Patient No.	Sex	No. blood samples	Pos.	Neg.	Amine content γ/cc.[2]	Remarks
14	♀	1	0	2	0	
21	♂	1	0	1		
24	♂	1	0	3	4	
30	♂	4	0	2	1.5	
33	♂	1	2	0	4	
35	♀	1	1	0	6.7	
36	♂	2	1	1	4	
37	♂	1	0	1		
38	♀	1	0	1		
39	♂	1	0	2		
40	♂	1	0	1		
41	♀	1	0	1		
42	♂	1	0	1		
43	♂	1	0	1		
44	♂	1	0	2		
Totals..............	15	19	4	19		
Per cent positive.....	18		13			

NOTE: Severe hypertension = diastolic pressure usually at levels of 130 mm. Hg or above. Moderate hypertension = diastolic pressure usually between 110 and 130 mm. Hg.

NOTE: Per cent positive: The first figure refers to the number of cases from which one or more extracts were positive. The second figure refers to the number of positive extracts.

[1] See figures 1 and 2.   [2] In terms of isoamyl amine of original blood.   [3] One sample taken after lumbo-dorsal sympathectomy.

Results According to Types of Subjects. a) Hypertensive patients. Material which raised the blood pressure in rats was found in a majority of the extracts made from the blood of hypertensive patients. In all, 46 extracts from 21 of the 23 patients showed pressor effects. These were demonstrated in samples taken at different times in 7 cases and in single samples in 13 (table 1). On the other hand, 30 samples were without effect. For example, 8 injections from

5 samples of arterial blood from the same patient were consistently inactive. Extracts from another case were active only in one of 4 injections from 2 samples. Extracts made from the blood of 3 patients yielded no pressor response.

In patients exhibiting renal diseases or involvement, the results presented a different picture (9). In 16 patients with little or no diminution of renal function, 39 extracts were active and 11 inactive.

Two samples of blood from another patient were active. A radical sympathectomy was performed and his blood pressure returned to normal. A sample of blood, taken several months after operation, possessed no pressor activity (fig. 4). In 5 hypertensive patients, classified as 'neurogenic,' extracts from 3 possessed little active pressor principle while that of one possessed none. In one case the results were doubtful. Only 7 of 26 injections of various extracts of blood from this type of case showed pressor substances in the rat. The blood of one patient, in whom pheochromocytoma was found at autopsy, was inactive.

b) Normal subjects. The extracts of blood from 4 subjects with normal blood pressures showed evidence of pressor substances. One of these subjects later developed arterial hypertension and another was suffering from congestive heart failure. There was no activity in extracts made from 18 others. Thirty-two injections of various types of extracts were inactive; 5 were active. Four samples of blood taken at different times from 1 subject and 2 from another showed no activity. In only one case were 2 injections of extracts active.

Results According to Types of Extracts. Fraction A. Pressor effects were demonstrated in 26 preparations of 31 different bloods from hypertensive patients, and only in 4 from 14 normal individuals. Of those suffering from renal hypertension, 29 of 31 injections were active, from neurogenic or other types, none of 10. This fraction therefore appeared to contain material giving more consistent results (fig. 4). Other fractions than *A* were studied to a smaller extent (figs. 5, 6).

The method of fractionation is presented in figure 1. The main fractions are emphasized there. Losses occurred during the fractionation. In several instances, when the first alcoholic filtrates were kept overnight in the cold before concentration, a slight precipitate formed. This material, collected in the cold and dissolved in saline, caused no characteristic pressor activity. A few tests seemed to indicate that there was little loss of activity at *Step 4*. This solution showed little or no pressor activity (*H* fraction). Pressor activity appeared in the *P* fractions. This represented material extracted by petroleum ether but which remained in solution in saline when the petroleum ether solution was freed of solvent in the presence of saline. It is possible that other material extracted by petroleum ether may have facilitated removal of the

active material. The characteristic activity did not appear to be associated with substances soluble in ether (*F* fraction). It apparently was lost when care was not taken to free the ether of peroxides.

When it appeared that active materials could be obtained from many hypertensive samples but few from normal ones, an attempt was made to discover whether or not the presence of pressor activity could be detected by means of a characteristic ultraviolet absorption spectra. For this purpose the *A* preparations (one cc.) were dried and re-extracted with 90 per cent alcohol to give the
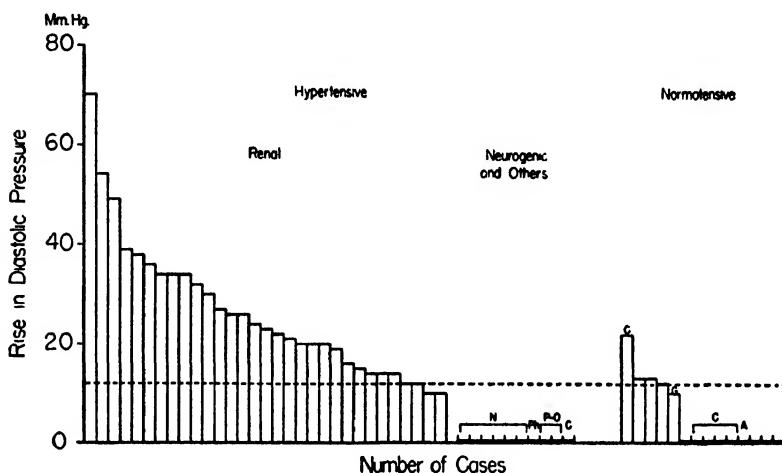
FIG. 4. Pressor effects of *A* fractions of arterial blood. Bars indicate sustained rise of diastolic pressure of rats measured 5 to 30 minutes after injection of an extract (usually at 15 minutes). All injections are shown. Dotted line represents a change of 12 mm. Hg, which is considered significant. *N* refers to neurogenic hypertension, *PH* to pheochromocytoma, *P-O* to post-operative, *C* to congestive heart failure, *G* to glomerulonephritis, and *A* to arteriosclerosis (see text).

D fractions. In a few instances the insoluble residue taken up in saline (*K* fraction) gave pressor effects as did the *X* fractions. While it appeared early that there might be a correlation between the presence of an absorption band at 2900 Å and pressor activity, additional samples made it evident that this was not the case. It was noticed, however, that the *D* fractions used for the ultraviolet absorption, when dried, redissolved in saline (*E* fraction) and injected into rats frequently gave a type of pressor response different from that originally present (fig. 5). It appeared to be more like that of the simpler amines. It is not clear whether or not the change resulted from additional handling of the material or depended specifically on the brief exposure to ultraviolet radiation. A modification in the activity of tyramine after exposure

to ultraviolet is recalled by this experience (10), hydroxy-tyramine apparently being formed.

A pressor activity at first similar to that of the E fractions but later sustained was observed in the picrates obtained in the estimation of amines. The picrates were collected, acidified, and freed of picric acid by extraction with peroxide-free ether. The resulting solutions showed some pressor activity in 9 of 12 samples from 11 hypertensive patients and in one of 5 from 4 normal individuals (fig. 6).
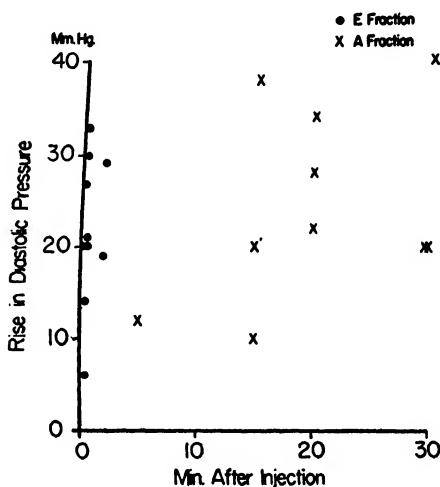
FIG. 5. *Left.* Change of time of pressor response of extracts exposed to ultraviolet light (E fraction). The crosses represent A fractions, the dots E fractions made from the same samples. The height of the activity of the latter occurred usually less than one minute after injection, and was soon over, as contrasted to the prolonged pressor response to the A fractions. All samples were from hypertensive patients.

Miscellaneous Observations. Although preparations were made, as a rule from arterial blood, pressor activity was obtained from venous blood in at least one instance. In several instances, amine picrates were formed. Very few experiments were made with separated components of blood. In some instances, the A fraction prepared from plasma was active; in others, it was obtained from cells of partially clotted blood. Adequate data on the distribution of pressor substances in blood are, however, lacking because whole blood was usually drawn directly into alcohol.

a) Depressor effects. Many of the extracts contained depressor material which may have masked some pressor activity. There was no correlation between primary depressor effects and subsequent appearance of pressor responses. Some of the fractions relatively free of depressor activity were the

picrates and the *P* and *E* fractions. The depressor material was insoluble in acetone.

b) Chromatographic adsorption. It was possible to effect some purification of the pressor extracts through absorption of the *A* and *D* fractions on an aluminum oxide column. The fractions were dried and dissolved in 10 per cent methyl alcohol-90 per cent acetone for adsorption. The major portion of the active material was eluted with mixtures of methyl alcohol and acetone, when the methyl alcohol content was between 20 and 50 per cent. Lack of material prevented more extensive use of this technique. It was not possible to compare the adsorption and elution of these fractions with angiotonin,
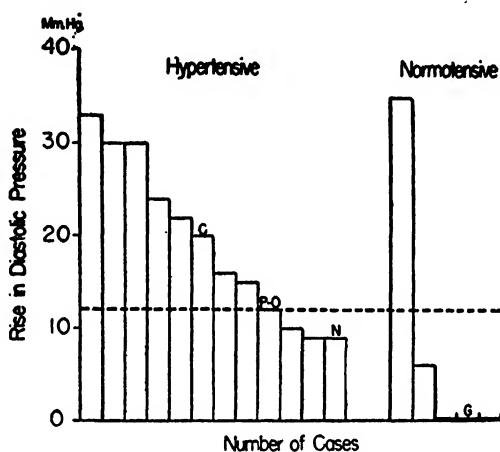
FIG. 6. *Right.* Pressor effects of picrates made from extracts of arterial blood. Notations same as figure 4.

for the sample of this substance available was found to contain a number of active pressor components.

c) Effects of amine oxidase on the activity. A number of the *A* fractions incubated in the Warburg apparatus with amine oxidase[1] showed a small absorption of oxygen. Afterward the material was no longer pressor. Material incubated under the same conditions without the enzyme retained its pressor effect. Likewise amine oxidase appeared to inactivate angiotonin and to cause a small absorption of oxygen. Similar tests with tyrosinase and *A* fractions of blood showed some loss in pressor activity though further loss might have been obscured by the presence of pressor material in the enzyme preparation. Similar amounts of oxygen were utilized by *A* fractions from normal and hypertensive patients.

[1] We wish to express our appreciation to Dr. A. Walti of Merck & Co., Inc., for supplying amine oxidase.

Effects of the Injection of Pure Compounds. Thirty-five injections of various substances were made in order to learn whether or not pressor responses similar to those seen after use of the active extracts of the blood could be produced. Similar results were not observed after the injection of angiotonin, tryptamine, isoamylamine, epinine, isovaleraldehyde, acetone soluble and insoluble fractions of an angiotonin preparation, casein hydrolysate, epinephrine and tyramine. One substance, phenylethylamine, did occasion a typical response in 4 of 7 instances. The addition of 100 γ of tryptamine to 2 A fractions resulted,
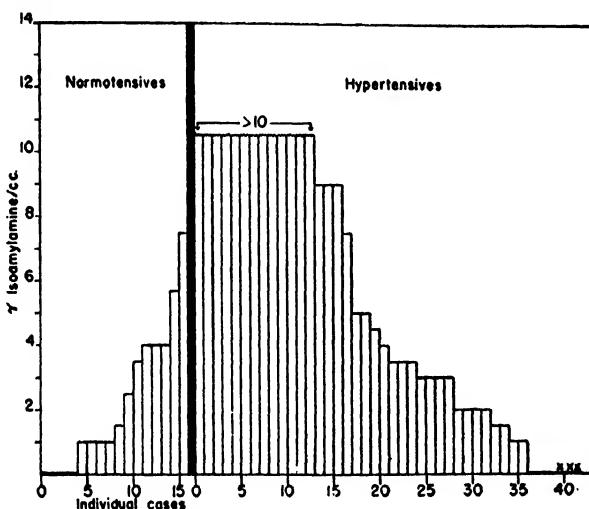
FIG. 7. Comparison of amine picrates, measured in terms of isoamylamine, in normal and hypertensive blood extracts. All extracts in which their color was estimated are included. The amount indicated is in terms of γ/cc. of original blood. The broken line indicates values which were too high to be read without dilution with the colorimeter at hand, that is more than 10 γ/cc. The samples marked X were those from which extraction was made with ether-containing peroxides.

after injection, in a state of shock, although the extracts contained little depressor material, and tryptamine was found to be a pressor substance.

Correlation Between Pressor Response and Intensity of Picrate Color. There was only a fair correlation between the presence of pressor material and the amounts of amine picrate (table 1). It was not good, possibly because the method of estimation did not measure all of the amines. Figure 7 shows diagrammatically the relative amounts of amine picrates present in the normal and hypertensive groups. It can be seen that the latter group tended to be higher.

Dogs' Blood. Blood extracted from 2 normal dogs was inactive. One sample

of 4 taken from a dog, made hypertensive, was inactive, but in 2 others an immediate pressor response was noticed.

DISCUSSION

It appears from the results described that when blood from hypertensive patients is extracted, according to the method outlined in this report, preparations capable of raising the blood pressure of normal rats are usually obtained. We conclude that pressor substances were present in the blood of most of the cases of arterial hypertension which were studied. It appears also that normal blood extracts were, for the most part, inactive.

It is noteworthy that the blood of over half of the hypertensive patients classified as 'neurogenic' yielded no active pressor material. This observation suggests either that pressor substances are present only intermittently and in small amounts or that some other mechanism underlies their hypertension. Further evidence in favor of this possibility has been gained (11). In patients with renal involvement or disease, the differences found are evidence that hypertension is a manifestation of a group of diseases.

The estimation of amines in extracts of blood suggested further that there might be more in hypertensive than in normal individuals. It should be emphasized that severe limitations in the method of estimation exist: 1) phenolic amines such as tyramine and epinephrine are not extracted in the procedure; 2) smaller amines such as the methyl amines do not produce a color; and 3) the color formed is not an accurate index of amine content as different amine picrates exhibit different transmission spectra. The picrates have accordingly not been considered accurate measures either of the total amines or of the pressor substances present in the blood or the extract. The method was used to learn whether or not an estimate of the relative amounts of measurable amines would indicate an underlying metabolic disturbance in the deamination of amino acids.

Evidence on the nature of the pressor substances in the obviously crude extracts is incomplete. From the preliminary observations it would appear that when acidified they are soluble in 90 per cent alcohol and insoluble in petroleum ether and in ether, though possibly inactivated by ether which contains peroxides. When alkalinized, some of the pressor material is extractable with toluene and forms picrates, from which pressor activity may be recovered.

Information gained from the method of preparation of the A fractions, from the estimation of amines and recovery of activity from the picrates, suggests that the pressor activity found in the majority of the cases of hypertension in this study is due to more or less complex amines. It is anticipated that further studies now in progress will elucidate this idea.

The methods used in this study should receive comment and criticism.

Anesthetized rats were found to make good test animals, as long as anoxia from respiratory obstruction did not occur. The sensitivity of these animals to the active material as compared with other animals or preparations was not tested. It may be that the sensitivity of rats can be increased by some procedure. At any rate, their blood pressure remains remarkably constant for relatively long periods of time, tending only to become lower. We have not noticed spontaneous elevation of blood pressure after the level has become established. The amount of fluid injected or the content of salts or other materials arising from preparing the materials probably did not affect the results. This belief is fortified by controlled observations with many other blood extracts, with hypertonic (6%) saline, with A extract containing 9 per cent alcohol and with relatively large amounts and volumes of various materials not reported here.

It has been suggested (12) that renin may have been liberated by the kidneys of these patients when relatively large volumes of arterial blood were removed and that the results may have resulted from the formation of angiotonin or hypertensin. We do not believe that this substance or its products account for the results observed. The pressor response of the active extracts was in no way similar to that seen after injection of angiotonin.

It is of considerable importance to discover the chemical nature of this pressor material. These preliminary observations merely point the way toward further study of this and similar substances; they may play a decided part in the mechanism of some forms of arterial hypertension.

SUMMARY

Arterial blood from hypertensive and normal patients was extracted and concentrated. The extracts were then injected into rats for the purpose of discovering the presence of pressor substances. In a majority of the extracts from 21 of 23 hypertensive patients, prolonged pressor effects were found. One fraction especially contained the active material. In a majority of the extracts from 22 normal individuals these pressor effects were absent. The amine content of the extracts of hypertensive blood was usually higher than those of normotensive blood.

This study offers evidence that blood from hypertensive patients contains prolonged pressor substances which are not present in most normal individuals. Possibly the substances are amines.

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LEPTOSPIROSIS IN CATTLE

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In 1948, Baker and Little[1] reported their study of an outbreak of bovine leptospirosis in a New Jersey dairy herd in 1946. Since then, extended observations on this herd, studies on other herds, and information reported by others have shown leptospiras* to be important agents in causing disease of cattle. Furthermore, there are found in the literature descriptions of clinical conditions of undetermined cause, principally hemoglobinurias, which suggest that leptospirosis may be even more widespread. Now that sufficient basic information has been accumulated by experimental procedure to provide accurate diagnosis and to apply preventive measures, it is felt worthwhile to present these findings, especially since the signs of illness may be so variable that, unless the practitioner is fully aware of the symptomatology, many outbreaks will be missed.

SYMPTOMS

In New Jersey, most outbreaks were observed from May to early November, although sporadic cases occurred at other times. Mild disease characterized by fever, albuminuria, and, in the case of lactating cows, bloody or thickened milk, was seen oftener, but occasional animals were severely affected and showed hemoglobinuria. Although both degrees of host reaction to a single strain of Leptospira were produced experimentally, it is considered helpful in presenting the clinical descriptions to discuss the mild and the severe forms separately.

Severe Form.—Usually, the severe form of infection is fatal within two to ten days. The onset is sudden or preceded by a day of inappetence and a drop in milk yield. During the acute phase, there are fever, depression, anorexia, dyspnea, and a marked reduction in milk yield. The temperature may vary from 103 to 107 F., and fever persists throughout the illness. In a day or so,

* The classification of organisms with spirals is most uncertain and confused. In the last edition of Bergey's Manual of Determinative Bacteriology (The Williams & Wilkins Co., Baltimore, 6th ed., (1948): 1051), they are all grouped under the order Spirochaetales, which is divided into two families, Spirochaetaceae and Treponemataceae. The former includes forms with many spirals and some internal structures, while the latter includes shorter forms with fewer spirals that are more tightly coiled. The aërobic forms in the latter family are in the genus Leptospira, and, therefore, the organisms that we are describing fall into this genus. The term "spirochete" is a more general one and is used for many of the forms in both families.

the visible mucous membranes become pale and icteric (yellow or orange). The milk from all quarters is bloody, with a pink, red, or brownish tinge, occasionally containing flecks of blood. The udder is soft and pliable, suggesting little, if any, irritation to the secretory tissue. This limp udder, resembling the gland of a dry cow, is of diagnostic significance. Hemoglobinuria is usually present, the color of the urine being bright red or dark brown. Pregnant animals are quite likely to abort early or during convalescence, and usually the placenta is expelled with the fetus. Before death, the symptoms become aggravated. In addition to prostration, the pulse and respiration are increased, the urine is bright red, and the feces are occasionally yellow. If severely affected cattle recover, the convalescence is prolonged due to continued fever, weakness, anemia, and nephritis.

Mild Form.—The mild form is a similar, but less severe, infection, rarely fatal, and lasting for two to four days. There may be depression, anorexia, dyspnea, abortion, and drop in milk yield; or the animal may appear normal except for a lowered production and a change in the character of the milk and urine. A fever of 102 to 105 F. persists for two to three days. The milk may be bloody, but it is more commonly thick, yellowish, and viscid. Blood in the milk may be detected only at the end of the milking, it may be seen at only a single milking, or it may persist for two or more days. Occasionally, the foremilk is bloody and the later milk is viscid and yellowish. The mammary gland is always soft and limp. Abortions do not occur as frequently as in the severe disease. Hemoglobinuria may occur early or as the cow recovers, and, invariably, the urine is dark brown.

In some cows, steers, and bulls, the rise in temperature and the hemoglobinuria are the only clinical signs of the infection observed. Occasionally, a mild case may die, and animals with hemoglobinuria may not fully recover for weeks.

In 1946, we had an opportunity to study a mild outbreak of leptospirosis in a mixed dairy herd in New Jersey,[1, 2] in which 105 cows had a bloody or a thick viscid secretion (58 and 47 cases, respectively) during a period of thirty-seven days. It is particularly significant that none of the affected cows died, aborted, or were icteric, and only 8 of the 105 animals had hemoglobinuria. The outstanding signs were fever, mild depression, and anorexia associated with a bloody or thick viscid secretion from a limp udder. Later, it was shown by the serologic examination of the blood that from 10 to 20 per cent of the cattle in some groups of 50 animals tested had inapparent infections not recognized by the veterinarian or the attendants, even though strict routine control measures were instituted in the herd to prevent the spread of the disease. After the outbreak had subsided in 1946, a few sporadic cases occurred in 1947, 1948, and early in 1949. Leptospira isolated each year from certain cases showed that the same antigenic strain was the etiologic agent. Furthermore,

bloody milk was a more constant symptom of the infection than hemoglobinuria or a thick viscid secretion from the udder.

Notes on Individual Cases

The following protocols of 3 natural cases of the disease in the cow not only portray the symptoms and course of the infection, but, in addition, present the results of the inoculation of guinea pigs with blood, milk, or urine.

Case 1.—A Guernsey cow calved for the fourth time on May 6, 1946. On Dec. 2, 1946, the animal showed the following symptoms: temperature 104.8 F., depression, anorexia, bloody milk from all quarters, udder soft and pliable. On December 3, her temperature was 103.8 F. and she showed inappetence, depression, drop in milk yield, and pinkish secretion. Samples of blood, milk, and urine were inoculated into 6 guinea pigs (blood −; milk +; urine −). On December 4, her temperature was 101.8 F., and her condition was the same: still depressed, milk pinkish, udder limp and flabby. Four guinea pigs were inoculated with milk and urine (milk +; urine −). On December 5, the temperature was 101.8 F., there was no improvement in condition, and six guinea pigs were again inoculated with blood, milk, and urine (blood −; milk −; urine −). The animal was isolated until December 23. During this period, the milk was abnormal (bloody or faintly tinged with blood) for fifteen days. The cow returned to production on December 25, and successfully completed the remainder of the lactation.

The milk of this cow was infective for guinea pigs on the first and second day of illness, while samples of blood and urine were negative. Blood drawn from this animal for serologic examination on December 3 was negative; whereas, a second sample obtained after recovery both agglutinated and lysed suspensions of the spirochete in high dilutions. The strain has been maintained in guinea pigs on serial passages, and a pure culture of the spirochete was isolated from the blood of guinea pigs.

Case 2.—A Holstein-Friesian cow, in milk when purchased in April, 1947, on Nov. 18, 1947, showed depression and inappetence. On November 19, the temperature was 106.5 F., and milk from all quarters was thick and viscid. The udder was soft and limp. Two guinea pigs were inoculated with blood and milk (blood +; milk +). On November 20, the temperature was 102.8 F., the cow appeared brighter, and her appetite was good. Small clots of blood were passed in the milk. On November 21, the temperature was 101.6 F., she appeared brighter, and her appetite was improving. The milk was thick and viscid with a faint pinkish tinge due to flakes of blood. She appeared normal on November 22, except that the secretion was thick, viscid, and slightly pinkish. On November 24, the condition of the milk was about the same. At this time, milk and urine were inoculated into guinea pigs (milk −; urine −). Both blood and milk from this cow were infective for guinea pigs on the

second day of illness, while the animal was febrile with a temperature of 106 F. On serologic examination, the blood was negative at the onset of the disease, but, after recovery, the serum agglutinated and lysed suspensions of the spirochete in a 1:20,000 dilution. Since November 19, the strain has been carried through 90 continuous passages in guinea pigs. A pure culture of the organism was also isolated from the blood of infected guinea pigs.

Case 3.—The case history of a Guernsey cow purchased in September, 1947, and which calved that same month, follows: November 20—temperature 104 F., slightly depressed, appetite fair, milk from all quarters thick and viscid, udder soft and pliable; November 21—temperature 102 F., brighter, appetite good, milk appeared normal; November 22—returned to production; November 24—temperature 100.6 F., hemoglobinuria, milk appeared normal. Two guinea pigs inoculated intraperitoneally with 1 cc. of urine developed a fever, and the disease is now in its nineteenth passage in guinea pigs. A pure culture of the Leptospira has been isolated from the blood of these infected animals.

DIAGNOSIS

A diagnosis of leptospirosis can be made by (1) the inoculation of cattle or laboratory animals with the blood, milk, or urine from cases of the disease; (2) the isolation of the organism in special mediums containing horse or rabbit serum; (3) the microscopic examination of tissue sections stained with silver, or the demonstration of the organism in the blood or tissues under dark-field conditions; and (4) the serologic examination of the blood from recovered cases.

Inoculation of the Natural Host.—In our previous report,[1] we described, in detail, a number of methods for the diagnosis of leptospirosis in cattle. It was shown that the spirochete could be recovered from the blood, milk, and, occasionally, the urine of cows during the febrile period. Moreover, when hemoglobinuria or albuminuria occurred, the organism was present in the urine for periods long afterward. The experimental infections produced in the natural host (subcutaneous or intranasal inoculations) showed all possible variations, from no illness to death. Thus, the clinical classification (severe and mild) is of little significance other than to demonstrate the host response to the same infection.

With adult cattle, besides fever, depression, drop in milk yield, and an occasional case of albuminuria, the secretion from the udder was thick, yellowish, and viscid. In lactating cows, the inoculation of infective material never produced bloody milk. In young calves, as a rule, the infection was acute and associated with albuminuria, or, occasionally, hemoglobinuria and death. On intranasal inoculation, some calves reacted; whereas, others showed no signs of illness. When these animals were later challenged with infective material (subcutaneous inoculations), all were immune.

Inoculation of Experimental Animals.—The spirochete[1] isolated in New Jersey is readily transmissible to guinea pigs, rabbits, embryonated eggs, and mice. When guinea pigs were inoculated intraperiotoneally with 1 cc. of defibrinated blood, abnormal milk (bloody or thick and viscid), or urine, a febrile reaction began three to five days after inoculation and persisted two to four days. Another characteristic feature of the disease in guinea pigs was the development of scattered petechial hemorrhages in the lungs, and minute white spots in the liver (cell necrosis), observed when the animals were killed. The lesions were more marked when the guinea pigs were autopsied after the fever had subsided.

If the guinea pigs were bled aseptically from the heart during the febrile period, and 1 cc. of defibrinated blood inoculated into normal guinea pigs, the infection could be maintained indefinitely by serial passage. The inoculations rarely resulted in the death of the guinea pig. When these animals are used in the diagnosis of leptospirosis in cattle, the absence of fever and lesions should not eliminate the spirochete as the causative agent, unless the results of other laboratory tests are also negative. Negative inoculations can be due to keeping material too long at room or refrigerator temperatures, with a resultant destruction of the spirochete. It is essential to inoculate the guinea pigs soon after the collection of blood, milk, or urine, preferably at the farm. Furthermore, unless the blood is obtained from cows that are febrile, and unless the milk is either bloody or thick and viscid, the inoculations may fail to produce infection. Likewise, when the urine from sick animals is abnormal in appearance, the spirochetes are more likely to be present in sufficient numbers to induce infection. Another fact that should not be overlooked is that some strains of bovine Leptospira have been shown to be nonpathogenic for guinea pigs. Bernkopf *et al.*,[3] in Palestine, failed to produce clinical signs of illness in guinea pigs with the blood, milk, or urine from natural cases of the disease in cattle, although this material was highly infective for the natural host.

The Isolation of the Spirochete.—The primary isolations are made by adding 0.5 cc. of infected guinea pig blood or allantoic fluid from infected eggs to cotton-stoppered glass tubes (160 mm. × 15 mm.) containing 5 cc. of Schueffner's[4] fluid medium with 0.5 cc. of sterile horse or rabbit serum. The cultures are incubated for five to seven days at 30 C. (94 F.) and then examined for growth by dark-field illumination. If leptospiras are found, transfers are immediately made to new mediums and the tubes incubated again. For the storage of positive cultures, the cotton stoppers are replaced with sterile rubber stoppers and the tubes kept at room temperature, preferably in the dark. Monthly transfers are made thereafter. Chang's[5] medium has been used with equally good results in cultivating these spirochetes.

In the diagnosis of the infection in natural cases of the disease in cattle, the spirochete may also be recovered by the centrifugation of urine from affected

animals at high speed (Pickles centrifuge—5 to 10,000 r.p.m.). In centrifuged samples, the bacteria and debris are thrown to the bottom of the tube, while the leptospiras remain in the supernatant fraction. The bacteria-free supernatant fluid can then be used for microscopic examination, animal inoculation, or direct culturing.

The Demonstration of the Spirochete in Tissue Sections and the Blood.—With more difficulty and less surety than by other methods, spirochetes can be demonstrated in tissues and blood. In four outbreaks of bovine leptospirosis reported in the United States, [6–9] a diagnosis was made after autopsy by the histologic examination of Levaditi's preparations of tissue sections from the liver or kidney. In two of the outbreaks, the inoculation of infective material into guinea pigs caused some response, and leptospiras were later demonstrated in stained preparations of liver or kidney tissue from these animals. Recently, Simons[10] reported that thedane blue (saponin-methylene blue solution addition compound: Negotiation Ltd., Münchenstein, Switzerland) synthesized by him, markedly simplifies all current thick-film methods for the demonstration of unicellular blood parasites in minimum concentrations. When a more detailed report of this method is published, it is possible that spirochetes may be detected more readily in the blood stream of cattle during an acute attack of the disease.

The Serologic Examination of the Blood.—Antibodies for the spirochete can be found in the serums of both experimental animals and cows recovered from the disease. Usually, the serums of cows bled at the onset of the infection fail to agglutinate or lyse suspensions of the spirochete, but two to four weeks later, the reaction of the blood is positive in a dilution of 1:200 or higher.

The technique employed for the isolation and maintenance of the organisms in pure culture for serologic studies in Schueffner's[4] fluid medium has been described by Bernkopf *et al.*[3, 11] and Bernkopf and Little.[12] Suffice it to mention here that both living and formolized cultures gave identical titers as a rule. Of the two methods, however, living cultures are more suitable to employ since spontaneous clumping, which not infrequently occurs with formolized cultures, is avoided. Small antibody concentration may not be detected with living cultures, since minor degrees of lysis are more difficult to read than a slight agglutination.

Both methods of testing for antibody, by lysis or microagglutination, require the use of live cultures. Obviously, a simpler method that would eliminate the continuous use of living cultures would be preferable. Recently, Musaev[18] reported his findings with the complement-fixation test, in which a lyophilized antigen was used. Further study will be necessary to determine whether the complement-fixation test is more efficient and less time-consuming than other methods for the serologic examination of the blood of cattle in this disease.

It is realized that the serologic test is a presumptive test which simply shows that antibodies for the spirochete are present in the blood of recovered or exposed cattle. When it is impossible to employ other laboratory procedures in the diagnosis of the infection in cattle, this test is very helpful. Titers of 1:20 or below can be regarded as suspicious, while complete agglutination or lysis in a 1:200 dilution is considered positive. The blood of normal cattle rarely, if ever, agglutinates or lyses suspensions of the spirochete in dilutions above 1:20, but when a reaction does occur in a 1:200 dilution, it is only weakly positive. In one survey, two self-contained herds, consisting of 18 and 21 animals, respectively, were bled. In one, no samples of the serum reacted, while in the other herd (21 animals), the serums from 2 adult cows (7 years old) that had completed five and six pregnancies, respectively, were weakly positive in a 1:200 dilution. These 2 cows were from an experimental herd maintained by the Institute and kept under range conditions in two large, tightly fenced-in enclosures. Although none of the cattle showed any evidence of the disease in 1946, it seems possible that at some time since birth they may have had mild, inapparent infections.

In 1945, Allam and Beck[14] reported their clinical observations on a condition of cattle, on farms near Philadelphia, of which nonspecific hemoglobinuria was the outstanding symptom. The disease was characterized by fever, jaundice, abortion, hemoglobinuria, and pink milk. The mortality was around 3 per cent, while the morbidity varied from 10 to 50 per cent in different herds. In April, 1948, 5 cows were bled, 3 of which recovered from the disease in 1946. The blood from 4 of the animals both agglutinated and lysed suspensions of the New Jersey strain isolated by Baker and Little.[1]

In the fall of 1947, Sutherland and Morrill[9] studied an outbreak of leptospirosis in a beef herd in Illinois. Two cows had died before the disease was brought to the attention of the College of Veterinary Medicine. A third cow sickened on August 4. This animal was dyspneic with a temperature of 104 F., while the visible mucous membranes were pale and icteric. The urine was distinctly brown. A diagnosis of leptospirosis was made by Levaditi's preparations from the liver of guinea pigs inoculated with material from this case. Later, another cow sickened but responded promptly to treatment with sulfanilamide and sulfathiazole supplemented with 20,000 units of penicillin. Dr. Morrill kindly sent us the serums from 30 animals that included the serum from the recovered case. The blood from 13 of the cattle agglutinated and lysed suspensions of the New Jersey strain of the Leptospira in a dilution of 1:200. In this herd, 12 animals had inapparent infections that were not recognized during the outbreak.

In June, 1948, a number of cows in a New Jersey herd, consisting of 45 milking animals, showed symptoms of leptospirosis. Two had hemoglobinuria,

the milk of 2 others was thick and viscid, while 3 cows aborted. The veterinarian was not called until June 17, when the outbreak had about subsided. One of us (R. B. L.) visited the farm on June 18, and found that none of the animals was acutely sick. The milk from 1 cow (temperature 101.6 F.) was slightly yellowish in color but not viscid. Two guinea pigs were inoculated at the farm with a composite quarter sample of milk from this cow and 2 other pigs with the urine from a cow that had hemoglobinuria two days previous to the visit. Infection was not established in any of the pigs inoculated. Thirteen cows were bled on August 8, including 2 controls and 3 animals that had aborted since June. The serums from 9 of the cows, including 6 that aborted during May, June, and July, reacted; whereas, the serums of the 2 normal cows and 2 animals that aborted late in July were negative.

In July, 1948, during a period of five days, 16 cows sickened in another New Jersey herd of 88 milking Holstein-Friesians. The cows were febrile (temperatures 105 to 107 F.), depressed, with inappetence, and a marked drop in milk yield. The milk from all quarters was thick and viscid, and the udders were soft and limp. The duration of the attack varied from ten to fourteen days. One cow aborted during the first two days of the illness. According to the veterinarian and the owner, none of the cows had bloody milk and none was jaundiced or passed hemoglobin in the urine. Of 23 cows bled on September 15, 6 were recovered cases. All but three of the serums lysed living suspensions of the spirochete. Two of the negative samples were from cows that were in pasture during the outbreak and did not mingle with the milking herd until September.

The serologic examination of the blood of recovered cattle presents a simple test for the diagnosis of bovine leptospirosis. The high incidence of inapparent infections detected by the serologic examination of herd samples of blood indicates that during an outbreak many supposedly normal cows contract the infection.

EPIZOÖTIOLOGY

The natural mode of transmission of the infection is not known. It has been suggested that ticks, insects, rodents, contaminated water, and the natural discharges from sick animals transmit the disease. Since, in New Jersey, outbreaks of leptospirosis in cattle occur more frequently during warm weather, there may be unknown vectors that serve as hosts to the spirochete from season to season. It is difficult to conceive how the organism could survive in this area either in water or in swampy pastures during the winter months, since it is so readily destroyed by refrigerator temperature. It is highly probable that the actual host for the organism from one season to another may be the bovine animal with a chronic infection (nephritis) often unobserved in a herd.

The introduction of new cattle into a herd and the movement of animals within a herd offer opportunities for mild or chronic cases of leptospirosis to mingle with healthy individuals. It has been shown experimentally by the writers[1] that the organism was excreted in the urine of a calf for a period of fifty-three days after the acute symptoms had subsided. Moreover, Bernkopf[11] reported the presence of the Leptospira in the urine of cattle four weeks after infection, while the organism was demonstrated histologically in the kidneys from an affected animal eight weeks after the infection was first recognized. In natural cases of the disease, there is still the possibility that the organism may be present in the urine for periods much longer than have been observed thus far. It seems likely, then, that mild cases of leptospirosis occurring in the winter might escape detection, but such animals could be responsible for the persistence of the infection in a herd and even for the introduction of the disease into other herds.

The spirochete is usually present in the blood, milk, and, occasionally, the urine during the period that the animal is febrile. Moreover, when hemoglobinuria or albuminuria (nephritis) occurs, the spirochete may be present in the urine for long periods. Thus, the blood, milk, and urine from clinical cases of the disease in cattle may be infective for the natural host and, in some instances, for guinea pigs, mice, and rabbits. According to Baker and Little,[1] the urine from animals excreting the organism may transmit the infection to normal cattle by nasal inoculation, which might explain the spread of the disease during an outbreak.

PREVENTIVE MEASURES

The epizoötiologic features thus far observed indicate that infection may be brought into a herd by carriers of the Leptospira. Control, therefore, should be directed toward the recognition of these carriers in order to prevent their introduction into herds of susceptible cattle. A suggested procedure follows:

1) Serologic Examination of Animals for Presence of Antibodies.--This would detect cattle that had had infection but would not indicate carriers, since antibodies would persist longer. Also, animals either in an incubation period or with acute infection would not show presence of antibodies; and, in order to detect these cases, it would be necessary to retest after a holding period of at least three weeks.

2) The urine of animals that show antibodies should be tested for leptospiras by the methods described under "Diagnosis."

Obviously, animals shown to be carriers should not be placed in contact with susceptible ones. No chemotherapeutic or antibiotic agent has been tested for elimination of leptospiras from carriers, but, should an effective one be found, it would simplify the problem and increase the reward for testing, since these animals could be salvaged.

The Classification of the Spirochete Isolated from New Jersey Cattle

The study of pure cultures of bovine Leptospira has shown that many strains not only differ in their pathogenicity for guinea pigs and mice but, occasionally, in their antigenic properties. Schueffner and Mochtar[15] reported that guinea pigs which had recovered from the inoculation of some particular strain were protected against challenge inoculations with other strains which antigenically had no relationship. Hence, it would appear that most cultures of bovine Leptospira interfere with reinfection by other strains, regardless of their serologic or pathogenic properties.

Fortunately, it was possible for us to compare the New Jersey culture with a strain that Bernkopf[12] had isolated from a natural case of the disease in a cow in Palestine. The culture was typical of the organism found there in cattle. The New Jersey and Palestine strains differed not only in their infectivity for guinea pigs but also in their antigenic properties. Nevertheless, the Palestine culture interfered with infection by the New Jersey strain in guinea pigs. Blood of recovered cattle from outbreaks occurring in New Jersey, Pennsylvania, and Illinois agglutinated and lysed suspensions of the New Jersey culture and reacted not at all, or in a very low titer, with the foreign strain.

In a more recent outbreak, in cattle in Pennsylvania (unpublished), two new strains were isolated and compared with the New Jersey culture. Cross-immunization experiments and serologic studies showed that these strains were identical. It would appear, therefore, that the bovine Leptospira responsible for outbreaks of disease in cattle in New Jersey, Pennsylvania, and perhaps Illinois is the same antigenic strain.

SUMMARY

Bovine leptospirosis is an infectious disease of dairy and beef cattle that may have existed in this country for some time, but was not recognized until 1944. It is a disease of importance to dairymen and stockmen, for, although the mortality may not be great, the losses in milk yield during an acute attack, or during a prolonged convalescence, and in the anticipated calf crop may be considerable. The clinical signs which would lead one to suspect this infection are described in some detail.

A positive diagnosis is made by the isolation of the Leptospira from infected guinea pigs or from embryonated eggs inoculated with blood from the guinea pigs. It is emphasized that inoculations be made with fresh material, since the Leptospira is very labile and may be lost if the material is chilled or kept any length of time. A presumptive diagnosis may be made by the finding of agglutinins and lytic antibodies in the serums of infected animals.

By serologic tests, it has been shown that the same antigenic strain of the

Leptospira was responsible for outbreaks in New Jersey, Pennsylvania, and Illinois, and that the strain causing a similar disease in Palestine differed in its antigenicity.

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AN OUTBREAK OF BOVINE LEPTOSPIROSIS IN PENNSYLVANIA

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Bovine leptospirosis was first recognized in this country in 1944 when Jungherr[1] reported three fatal cases in dairy cows in Connecticut. Since then spirochetal‡ infections in cattle have been observed by Marsh[2] in Montana, Mathews[3] in Texas, Baker and Little[4] in New Jersey, ꞌd Sutherland and Morrill[5] in Illinois. In four of these reports, a diagnosis was made by the demonstration of the organism in stained tissue sections of the liver and kidneys from guinea pigs inoculated with infective material or directly from the organs of natural cases which had died. Baker and Little[4], on the other hand, isolated pure cultures of the organism from the blood of guinea pigs inoculated with infective material from sick cattle. More recently these authors[6] reported in greater detail the clinical signs of the disease in cattle and reviewed the practical application of a number of tests which can be employed for the diagnosis of the infection. They showed that the blood, milk, and occasionally the urine, are infectious for the natural host and readily cause signs of illness and lesions in guinea pigs and hamsters. Moreover, it was pointed out that at the onset of the disease, the blood of cattle usually fails to agglutinate or lyze suspensions of the Leptospira, but following recovery these tests are positive. As a routine diagnostic measure, it was recommended that all infected cattle be bled during the acute phase of the disease and again after recovery and that these serological tests be made. When facilities are not available for the direct examination of material from affected cases, a retrospective diagnosis can be made by the serological examination of the blood for the presence of agglutinins against the Leptospira.

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‡ The classification of organisms with spirals is most uncertain and confused. In the last edition of Bergey's Manual of Determinative Bacteriology (The Williams & Wilkins Co., Baltimore, ed. 6 [1948]:1051) they are all grouped under the order Spirochaetales, which is divided into two families, Spirochaetaceae and Treponemataceae. The former includes forms with many spirals and some internal structures, while the latter includes shorter forms with fewer spirals that are more tightly coiled. The aerobic forms in the latter family are in the genus Leptospira, and therefore the organisms (Fig. 1 and 2) that we are describing fall into this genus. The term "spirochete" is a more general one and is used for many of the forms in both families.

Apparently an unrecognized outbreak of bovine leptospirosis occurred in Pennsylvania in 1945, for Allam and Beck[7] reported their clinical findings on a condition of cattle in which the most outstanding symptom was a non-specific hemoglobinuria. This abnormality occurred in herds of Philadelphia, Chester,

FIG. 1. Preparation of spirochetes (×1300) from culture stained by Morosow's method. (Reprinted from Baker, J. A., and Little, R. B., *J. Exp. Med.*, 1948, **88**, 295.)
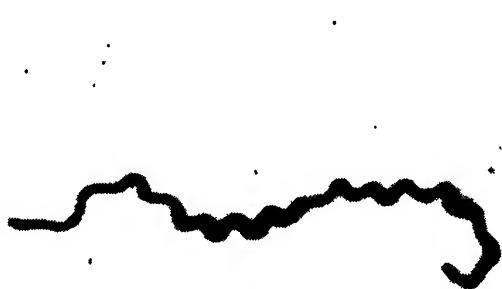
FIG. 2. Electron microphotograph of spirochete (×19,750) that shows usual unit of size, 90 millimicrons in diameter and 4 microns long. (Reprinted from Baker, J. A., and Little, R. B., *J. Exp. Med.*, 1948, **88**, 295.)

Bucks, Delaware, and Montgomery counties in Pennsylvania during the summer and fall of 1945. The disease was characterized by hemoglobinuria, severe anemia, slight icterus, fever, pink milk, decreased milk production, abortion, moderate constipation, anorexia, and loss in weight. The morbidity varied from 10 to 50% in different herds, while the mortality rate was around 3%. In April of 1948, five cows in one of the herds involved in the Chester county outbreak

of 1945 were bled. The sera from three animals had specific antibodies for the Leptospira isolated by Baker and Little[4] from sick cattle in New Jersey. This was the first indication that possibly a Leptospira was the etiological agent in this outbreak.

The purpose of this presentation is to describe an outbreak of bovine leptospirosis which occurred in Pennsylvania during the summer and fall of 1948 in which agglutinins were demonstrated in the blood of recovered cattle and the organism was isolated from blood or milk of two affected animals on different premises. The disease was recognized on seven farms, involving 33 head of cattle.

History of the Outbreaks

The disease did not recur in Pennsylvania until July of 1948. Since the symptoms presented by the sick cattle were similar to those observed by Allam and Beck[7] in 1945, a tentative diagnosis of bovine leptospirosis was made. In order to obtain more information regarding the epidemiology of the disease occurring in Chester and Montgomery counties, one of us (J. V. McC.), a practitioner, instructed his clients to take the temperatures of all sick cattle, including cows with thick milk in which the udders were soft and limp. Furthermore, they were asked to observe the urine for the presence of hemoglobinuria. With this cooperation it was possible to obtain fairly accurate histories and to treat the animals promptly as they sickened. The general symptoms in the affected cattle were fever, anorexia, depression, abortion, and a thick, viscid, yellowish secretion from a soft and limp udder. Hemoglobinuria was observed in over 50% of the clinical cases.

From early in August through September, all attempts to establish the infection in guinea pigs and hamsters failed, so that the diagnosis of leptospirosis was questioned. In no instance, however, was material obtained for inoculation from a sick animal with a temperature of 103 F. or above.

It was decided that, before any further investigation of this disease of cattle was carried on, the blood of recovered animals should be examined serologically to determine if agglutinins were present in their sera for the bovine Leptospira. On September 30, a group of 19 cows, two heifers, and two calves which had recovered from the disease (six farms) were bled. The results of the agglutination test are submitted in table I.

As can be seen in table I, 19 of the 23 sera examined gave positive results, indicating that the blood of these animals contained specific agglutinins for the spirochete. It is of particular interest that a previous sample of blood obtained from cow 15, herd V, at the onset of the disease was negative, but a second sample obtained (21 days later) at the time the recovered cattle were bled, reacted in 1:20,000 dilution. The results of the serologic test suggested that the etiological agent in this disease was a Leptospira. Obviously, in order to confirm

the diagnosis, it seemed necessary to isolate the organism from sick cattle. Early in October, eight guinea pigs from the Princeton stock were sent to Doctor McCahon to be taken to the farms for inoculation whenever new cases developed. Immediately after the guinea pigs were injected at the farm they were to be expressed to the Institute so that daily temperatures could be taken. Doctor McCahon was advised not to inoculate these guinea pigs unless the affected cattle were acutely sick with temperatures of 103 F. or above.

The results of the animal inoculations and the clinical and serological findings in each herd will be discussed separately in the order presented in table I.

Herd I

A purebred Guernsey herd consisting of 35 animals: Early in August a cow became ill, presenting typical signs of the disease. The udder was limp and the milk thick and viscid. On November 17, another cow showed similar symptoms. The temperature of this animal was 104.6 F. Two guinea pigs from the Institute stock were taken to the farm and inoculated intraperitoneally with 1 ml. of whole blood and 1 ml. of thick viscid milk, respectively, from this animal.

The guinea pigs were immediately expressed to Princeton where daily temperatures were taken. Both guinea pigs became febrile on the fifth day and were bled on the following day. A pure culture of Leptospira was isolated from the blood of these guinea pigs and the organism is being maintained by serial passage in guinea pigs.

The cows were treated and both recovered.

Although only two clinical cases were observed in this herd during the entire season, the serological examination of herd samples of blood showed that 11 additional animals had unapparent infections.

Herd II

A small mixed herd (29) of Jersey, Holstein, and Guernsey cattle: In August, cow no. 2 sickened. The symptoms were fever, hemoglobinuria, anorexia, and a thick, viscid, yellowish secretion from a limp udder. She aborted during the early course of the disease. Cows no. 3 and 4 were not severely ill, yet each animal aborted. Another cow (no. 5) calved prematurely, but there was no history that she had been sick. Of the four sera tested from this herd, the only negative sample was the serum from cow no. 5.

The serological examination of herd samples of blood showed that, in addition to the four recognized cases, 12 other animals had unobserved infections.

Herd III

A small purebred Guernsey herd in which two animals were severely affected: One cow died, while the other, a hopeless case, was destroyed. Shortly before the cow died (temperature subnormal), a sample of citrated blood was inocu-

lated into two hamsters and two guinea pigs. The symptoms in both cows were fever, jaundice, hemoglobinuria, and bloody milk. An autopsy was performed at the University of Pennsylvania Veterinary Hospital on the cow that was destroyed. The outstanding lesions were jaundice, petechiae on

TABLE I

The Results of the Serological Examination of the Blood of Recovered Cattle

No. of Herd	No. of Animal	Lysis			
		1:20	1:200	1:2,000	1:20,000
I	C* 1	+**	+	−	−
II	C 2	+	+	+	+
	C 3	+	+	+	−
	C 4	+	+	+	−
	C 5	−	−	−	−
III	C 6·	+	+	+	±
	C 7	+	+	+	±
	C 8	+	−	−	−
	C 9	+	+	+	+
IV	C 10	+	+	+	−
	C 11	+	+	+	−
	H 12	+	+	+	+
	H 13	+	+	+	+
V	C 14	+	+	+	±
	C 15	+	+	+	+
	C 16	+	+	+	±
	C 17	+	+	+	+
	C 18	+	+	+	±
	C 19	+	+	+	−
	C 20	+	+	+	+
VI	C 21	+	+	+	+
	Ca 22	±	−	−	−
	Ca 23	−	−	−	−

* C = cow, H = heifer, Ca = calf.
** + signifies all organisms lyzed; ±, many organisms lyzed; −, no lysis.

surface of epicardium, bloody pericardial fluid, and subcapsular hemorrhages in both kidneys.

The day after this cow was destroyed, a stallmate sickened. This latter animal was treated promptly and made a complete recovery.

Herd IV

A large purebred herd of Guernsey cattle maintained in separate units on three different farms: A single cow in two of the units and two heifers at the young stock farm had the disease. The symptoms in the cows were typical, while the chief signs observed in the heifers were fever and hemoglobinuria. These four animals were the only clinical cases observed on this stock farm, consisting of 50 cows and young stock.

Herd V

A purebred herd of Holstein cows of over 40 milking animals: In July, the first case of leptospirosis was recognized in a cow showing typical signs of the disease. From July to early in August, three more cows became ill. On August 10 cow no. 15 sickened. The owner stated that on the previous day a few small clots of blood were detected on the milk strainer pad. The symptoms were anorexia, mild depression, and a yellowish, watery secretion from a limp udder. The temperature was 102.8 F., and the urine appeared normal. Six guinea pigs transported from Princeton were inoculated with the blood, milk, and urine. None of the pigs became sick and, when challenged three weeks later with the New Jersey culture, all reacted. A sample of blood from this cow on August 10 was negative; whereas sera obtained 21 and 81 days later, respectively, had titers of 1:20,000. Of the seven clinical cases of the disease, hemoglobinuria was observed in only two cows. Forty milking cows in this herd were bled on November 30, and the sera from only 20 animals failed to lyze suspensions of the spirochete. In addition to the seven clinical cases, 13 other cows apparently had mild unapparent infections not detected by the owner.

Herd VI

Small herd of purebred Guernsey cattle: In September, a typical case of the disease was observed in a cow in milk. The animal was febrile, depressed, off feed, with hemoglobinuria. The milk was thick and viscid. On September 31, this cow and two young calves, no. 22 and 23 (three to four weeks of age), were bled. The young animals had a history of hemoglobinuria a few days prior to the visit. Samples of blood obtained from these calves at this bleeding and 60 days later failed to lyze suspensions of the spirochete. Since the disease had occurred in an adult animal in this herd (blood + 1:20,000), it seemed logical to assume that the hemoglobinuria in the calves could be attributed to the same etiological agent, regardless of the negative reaction of their sera. It is realized that young animals under four months of age may not be capable of forming antibodies in their blood for specific microorganisms until they become more mature. In another disease of cattle, caused by *Brucella abortus,*

repeated inoculations of living cultures of the organism may fail to initiate any response in the blood of the young calf to the production of antibodies. Apparently, young animals that are immune fail to show agglutinins in their blood.

Herd VII

This herd was not included in table I, for on the day the recovered cases were bled, the farm was not visited on account of distance. In August, a cow died which had shown typical symptoms of the disease. This animal was not treated. Later six other cattle, including a bull, contracted the infection. This latter animal sickened on November 1 with a temperature of 107 F., depression, anorexia, and hemoglobinuria. It was treated promptly but did not fully recover until ten days later. Guinea pigs were inoculated intraperitoneally with 1 ml. of whole blood from this animal at the onset of the disease. They were expressed to Princeton and on the sixth day became febrile and were bled from the heart. At this bleeding a pure culture of the Leptospira was isolated from their blood, and since then the organism has been maintained in guinea pigs by serial passage. This herd was kept under very poor sanitary conditions, so it was felt that the serological examination of herd samples of blood would reveal a high incidence of unapparent infection. This deduction was not substantiated, for of the 21 adult animals and two yearlings tested, only four sera, in addition to the blood of the six recovered cases, had positive titers.

Comparison of Strains of Leptospira Isolated from Cattle in New Jersey and Pennsylvania

It has been demonstrated[4] that the Leptospira isolated from cattle in New Jersey caused typical signs of illness and lesions in guinea pigs. Fever developed three to five days following the inoculation of infective material, and at autopsy, characteristic lesions were encountered, such as scattered petechial hemorrhages in the lungs and small whitish spots in the liver (cell necrosis). Occasionally, subcapsular hemorrhages appeared in the kidneys. The inoculations rarely resulted in the death of the guinea pig and recovered animals were solidly protected against challenge inoculations with infective material.

The two strains of Leptospira isolated from Pennsylvania cattle produced reactions and lesions in guinea pigs undistinguishable from those already observed.[6] Guinea pigs which had recovered from the inoculation of blood or milk from affected cattle in Pennsylvania were protected against the reinoculation of homologous strains. Cross-infection experiments in guinea pigs with both the Pennsylvania and the New Jersey strains showed that one culture would fully protect against a challenge inoculation with the other

strain. Moreover, when sera from recovered cattle were tested by agglutination, both strains had the same limiting titer.

Bernkopf and Little[8] compared the New Jersey organism with a typical

TABLE II

Lysis Tests with Immune Sera of Cattle Against the New Jersey, Pennsylvania, and Palestine Strains of Leptospira

Location of Cattle	No. of Herd	No. of Cow	New Jersey				Pennsylvania				Palestine			
			1:20	1:200	1:2,000	1:20,000	1:20	1:200	1:2,000	1:20,000	1:20	1:200	1:2,000	1:20,000
New Jersey	I	1	+*	+	+	+\	+	+	+	+	−	−	−	−
		2	+	+	+	+	+	+	+	+	−	−	−	−
		3	±	±	+	+	+	+	+	+	−	−	−	−
	II	1	+	+	±	+	+	+	±	±	±	−	−	−
		2	+	+	±	+	+	+	±	±	−	−	−	−
	III	1	+	+	+	+	+	+	+	+	−	−	−	−
		2	+	±	+	+	+	+	+	+	−	−	−	−
		3	+	+	+	+	+	±	+	+	−	−	−	−
	IV	1	+	+	+	+	+	+	+	+	−	−	−	−
Pennsylvania	II	1	+	+	+	+	+	+	+	+	−	−	−	−
		2	+	±	+	+	+	+	±	±	−	−	−	−
	III	1	+	+	+	±	+	+	+	±	±	−	−	−
		2	+	+	+	+	+	+	+	+	−	−	−	−
		3	+	±	+	+	+	+	+	+	−	−	−	−
	IV	1	+	+	+	+	+	±	+	+	−	−	−	−
		2	+	+	+	+	+	+	+	+	±	−	−	−
	V	1	+	+	+	+	+	+	+	+	−	−	−	−
		2	+	±	+	+	±	±	±	+	−	−	−	−
		3	±	+	+	+	+	+	+	+	−	−	−	−
	VI	1	+	+	+	+	+	±	+	+	−	−	−	−
	VII	1	+	+	+	+	+	+	+	+	−	−	−	−
		2	+	+	+	+	+	+	+	±	±	−	−	−
		3	+	+	+	+	+	+	+	+	−	−	−	−

* See footnote to Table I.

Leptospira culture isolated from an infected cow in Palestine. The two cultures differed not only in their pathogenicity for guinea pigs but in their antigenic properties as well, indicating that the Palestine strain belonged to another serological group.

The sera from a number of recovered cattle from New Jersey and Pennsylvania were tested by agglutination against their respective strains and the Palestine culture. The results of this test are given in table II.

It will be noted in table II that the sera from 23 recovered animals (Pennsylvania and New Jersey) reacted in about the same dilutions with both strains but not at all, or in a low titer, with the Palestine culture. Thus, it would appear that the New Jersey and Pennsylvania strains of Leptospira belong to the same antigenic group.

Treatment

In outbreaks of bovine leptospirosis, no experimental data are available concerning the value of treatment with drugs and various antibiotic agents in which treated animals and controls have been studied during the natural course of the disease. For this reason, it is not possible for us to evaluate the results of treatment with penicillin and sodium sulfathiazole administered by one of us (J. V. McC.) in the outbreak occurring in Pennsylvania in 1948.

In Weil's disease[9], (spirochetal jaundice in man; new term leptospirosis) transfusion with whole blood from donors who have recovered from the disease is considered the best single step in the treatment of a patient severely ill. On the other hand, it has been shown that in this disease the various sulfonamide compounds are of little value, whereas penicillin has a lethal as well as an inhibitory effect on the multiplication of the Leptospira. Moreover, when the parasite is being shed in the urine, the administration of penicillin has been very effective in eliminating the organisms from the kidney.

It has been suggested by Little and Baker[6] that chronic cases of the disease in cattle may serve as natural carriers of the infection. Thus, cattle may be actual vectors and hosts of the infection from one season to another, for it has been shown[4] that animals with nephritis shed the organism in the urine for long periods. When sick cattle are no longer febrile and the acute signs of the disease have subsided, they may not be carriers of the infection unless there is hemoglobinuria or albuminuria. Hence medication should be administered not only to hasten recovery but to eliminate carriers of the infection, for bovine leptospirosis is not only an economic disease of livestock but one of public health significance since some types of the bovine organism have been shown to be infective for man[10, 11]. Obviously, general treatment should be directed toward the control and eradication of this disease of cattle and therefore the following suggestions may be worthy of consideration:

1. The herd should be quarantined and each affected animal isolated during the actue stages of the disease and for at least a week following recovery. In addition, daily temperatures should be taken and each sick animal should be observed carefully for jaundice, hemoglobinuria, abortion, and changes in the character of the secretion from the udder.

2. The transfusion of whole blood (500 cc. or more drawn into sodium citrate)

from donors that have had the disease should be beneficial*. Moreover, whole blood from normal cattle may be used when donors are not available, to replace the red blood cells and other constituents'of blood depleted by the disease. The transfusion of whole blood can be repeated whenever necessary.

3. The administration of penicillin from the first day of illness is recommended. Other antibiotics and drugs should be tested for the elimination of carriers.

4. Since kidney damage (hemoglobinuria, etc.) frequently occurs, an acid urine may retard the multiplication of the Leptospira in the kidney. The daily administration of methenamine and sodium biphosphate while the animal is sick and for at least ten days after recovery, may maintain an acid condition of the urine during the critical period.

5. The effectiveness of any line of therapy is enhanced if the treatment is administered soon after the first symptoms are recognized.

<div align="center">DISCUSSION</div>

In bovine leptospirosis, the signs of illness in cattle may be so variable that many outbreaks may be missed unless the practitioner is fully cognizant of the symptomatology. In either the severe or mild form of the disease, the onset is sudden. Frequently the owner states that on the preceding day the cow was slightly depressed with inappetence and a drop in milk yield.

The severe form, in which animals are depressed, often prostrated with fever, associated with icterus, hemoglobinuria, pink milk, and abortion, should be less difficult to diagnose than the mild form of the disease in a herd where only a single cow or two during the entire season becomes ill. As a rule, in mild outbreaks, fever and depression of short duration in cattle are often attributed to indigestion or to some unknown condition but rarely to this disease. If cows which are depressed, with anorexia and fever, are carefully observed by the dairyman, other symptoms of clinical significance may be recognized to assist in the diagnosis. With the exception of fever, depression, and a marked drop in milk yield, the most consistent symptom observed by one of us (J. V. McC.) was a thick viscid secretion from a limp udder resembling the gland of a dry cow. Three of the affected cows had bloody milk; two of them died, while the third animal, a hopeless case, was destroyed. These were the only cattle that were icteric or had a typical bloody secretion from the udder.

In previous work on leptospirosis in cattle in a New Jersey herd, bloody

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* The disease appeared again in this section of Pennsylvania during the summer and fall of 1949, yet none of the dairy herds affected in 1948 were involved in this outbreak. One of us (J. V. McC.) transfused a number of acutely sick cattle with whole citrated blood (500 cc. or more) obtained from dairy animals that had shown clinical signs of the disease in 1948. This form of medication was the most satisfactory treatment employed thus far, since the natural course of the disease was shortened.

milk was a more constant abnormality than a thick viscid secretion. It would seem, therefore, that in this disease the milk from a limp udder may be either bloody or thick and viscid. Hemoglobinuria, a characteristic feature of the infection in young cattle, did not occur as frequently in adult animals. Abortion, another sign of the disease, may occur while the animal is sick or after recovery.

In the outbreak of leptospirosis in Pennsylvania, a tentative diagnosis was first made by the serological examination of the blood of recovered cattle. Later the diagnosis was confirmed by the isolation of the organism from the blood or milk of two clinical cases situated on different farms. These strains were similar in their pathogenic and antigenic properties to the organism isolated in a New Jersey dairy herd, and obviously the same serological strain responsible for an outbreak of the disease in the New Jersey herd in 1946 was the etiological agent in outbreaks occurring in Pennsylvania during the summer and fall of 1948.

The cultures isolated from cattle in New Jersey and Pennsylvania can be regarded as a typical bovine strain, since at present there is no indication that the organism is pathogenic for man. On the other hand, the bovine Leptospira isolated from cattle in Palestine[10, 11] is also pathogenic for man. Outbreaks occurring in this country should therefore be regarded as dangerous to human beings, and the herds quarantined until the organism is typed.

SUMMARY

A report is submitted of an outbreak of bovine leptospirosis that occurred in Pennsylvania on seven different farms during the summer and fall of 1948.

A positive diagnosis was made by the serological examination of the blood from recovered cattle and the isolation of the Leptospira from guinea pigs injected at the farms with blood or milk from natural cases.

By serological tests and cross-examination experiments with guinea pigs, it has been shown that the strains from Pennsylvania and New Jersey belong to the same antigenic group.

It is pointed out that at present there is no evidence that the bovine Leptospira isolated from cattle in New Jersey and Pennsylvania is pathogenic for man. Since some strains have been responsible for disease in human beings, any outbreak in cattle should be regarded as potentially dangerous to man until the organism is classified.

APPENDIX

While this outbreak of bovine leptospirosis was being studied, one of us (J. V. McC.) observed three horses that were jaundiced, febrile and weak. In two of the animals, the eyes were swollen and the pupils cloudy, with a marked watery lacrimation, followed by blindness. Since the signs of illness shown by the three horses were similar in many respects to those observed in

cattle, a tentative diagnosis of leptospirosis was made. After the horses recovered from the acute illness, they were bled and their sera reacted in a 1:200 to 1:2,000 dilution with both the New Jersey and Pennsylvania strains but not at all with the Palestine culture.

Since it seemed likely that horses in contact with cattle might contract leptospirosis, samples of blood were obtained from 29 horses from six different dairy farms. The sera from eight reacted in high dilutions (New Jersey and Pennsylvania cultures), yet none of the animals had a previous history of any infections resembling the illness observed in the three horses noted above.

From a large stud farm where blindness and abortion are frequent, numerous samples of sera from brood mares and colts have been tested for immune bodies against the leptospira. Many of these sera were positive in high dilutions to the New Jersey and Pennsylvania strains, and some agglutinated the Palestine strain in low dilutions. There is a close correlation between the results of the serological tests and the presence of the clinical abnormalities noted, but some of the positive animals have shown no such abnormalities.

So far the few attempts to isolate the organism from horses have been negative. These studies are being continued, for, in agreement with *Heusser, et al.*[12], it is recognized that the positive serological results do not prove that the Leptospira is the cause of the abnormalities noted.

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ASSOCIATION OF A SPECIAL STRAIN OF PLEUROPNEUMONIA-LIKE ORGANISMS WITH CONJUNCTIVITIS IN A MOUSE COLONY

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PLATE 15

Manifestations of ocular irritation, indicative of conjunctivitis, are not infrequently observed in experimentally infected mice. The interpretation of this reaction is handicapped by the lack of information concerning its occurrence in animals maintained under normal conditions. An extensive and persistent outbreak of conjunctivitis in a colony of white mice afforded ample material for study and led to the present inquiry into its pathogenesis and epidemiology.

The Distribution and Manifestations of Conjunctivitis in the Colony of Infected Mice

Manifestations of conjunctivitis were first observed during the winter of 1947 in experimentally infected mice of the Princeton strain (non-Swiss). Inspection of the breeding colony, which numbered approximately 750 mice, revealed similar signs of ocular involvement in a high percentage of the recently weaned young. The newly born mice are held in the breeding cages until the 3rd week of life when they are weaned and segregated in groups of 20 according to sex. Through about the 5th week after birth the number of mice of both sexes which showed evidence of conjunctivitis at a given time varied from 20 to 80 per cent. Periodic examination indicated that spread of the disease was progressive and that most of the young mice were ultimately affected. The acute signs of conjunctivitis began to abate as the mice matured and by the 10th to the 12th week of life many of them appeared normal. In some animals, however, the reaction was persistent and was recognizable after a period of months.

In most instances even the acute signs of conjunctivitis are far from striking and unless specifically looked for might readily be missed. The eyelids are somewhat swollen and may be coated with a sticky exudate which tends to glue them loosely together. If the lids are open there is an increased amount of fluid, which may be clear or turbid. The volume of fluid which can be removed on aspiration is definitely increased in comparison to that removable from a normal mouse. The hair adjacent to the eye may be wet and matted, resulting in an encircling ring. Occasionally there is a slight encrusting with blood. During the acute period of the disease both eyes are commonly involved.

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Two complications may occur but are relatively uncommon, the estimated incidence not exceeding 1 per cent. A few mice in the presence of acute conjunctivitis show prominent, nodular, subcutaneous swellings adjacent to the eye. This lesion has been observed only in young mice and always restricted to one side. An occasional mouse may show involvement of the eye itself which is distended outward with fluid. It usually becomes opaque and vision is lost.

The inflammatory reaction is limited to the eye and its appendages. In the absence of other infections there are no additional indications of illness, and at autopsy the respiratory tract, middle ears, and visceral organs are uniformly normal.

The Bacteriological Examination of Exudate from the Eyes of Infected Mice

Young Princeton mice showing obvious signs of conjunctivitis were removed directly from the breeding colony and anesthetized with ether. The tip of a capillary pipette was inserted beneath the eyelids and any fluid adherent to the conjunctiva and the outer surface of the eye was removed by suction. Air-dried films were prepared from the exudate after mixing it with a drop of water on a glass slide. These films were stained by the Gram method and also with the polychrome Wayson stain (1). This stain, which affords a color contrast, proved to be particularly useful and was employed as routine.

The first slides studied showed minute spherical bodies which stained blue with the Wayson stain and were Gram-negative. They were arranged in varying-sized, extracellular clumps and were also regularly present in or on the large, conjunctival, epithelial cells. More than 100 weaned mice with conjunctivitis have now been examined and all have shown similar bodies in exudate from the eye.

There was a considerable variation in the number of the bodies from animal to animal but in general they were numerous and readily found. Within the epithelial cell they were most commonly arranged in small clumps or distributed as discretely spaced particles over much of its surface (Fig. 1). There were also observed, at times, much larger intracellular structures which were circular or irregularly shaped and stained blue with the Wayson stain (Fig. 2). Some of these structures were compact and undifferentiated while others were granular in appearance and showed small discrete particles breaking away from the periphery. There is little question that they represent colony-like aggregates of the minute bodies. Polymorphonuclear leucocytes were commonly present, though generally few in number, and in some the small particles were clearly visible. The films also showed miscellaneous bacteria among which Gram-negative bacilli predominated and in some exudates were very numerous. These bacteria were not specifically identified.

The morphologic appearance of the minute bodies, particularly their variable arrangement within epithelial cells, was suggestive of an agent of the trachoma-infectious blennorrhea group. The outcome of cultural studies soon indicated, however, that the resemblance was purely superficial.

Conjunctival exudates were inoculated on a solid medium by expelling the contents of a capillary pipette into a drop of sterile saline solution which was then rubbed over its surface. The inoculated plates, which previously had been chilled in the refrigerator, were sealed with Scotch tape and incubated at 37°C. for 5 to 7 days. The medium which was regularly used was

approximately 30 per cent horse serum–nutrient agar containing 2500 units of commercial penicillin (0.25 cc. of a stock 10,000 unit saline solution of potassium penicillin G).

The first exudates cultured showed small colonies barely visible without magnification. In subsequent examinations similar colonies were regularly obtained from exudates in which the minute bodies were observed microscopically. Practically all the inoculated plates showed a pure growth of these colonies, which were characteristic and uniform in appearance. In some instances a few large colonies, generally molds, were also present. A diffuse growth of minute, pleomorphic, Gram-negative bacteria was regularly obtained in horse serum–bouillon on transfer from the small colonies. These bacteria were undoubtedly identical with the minute bodies observed in films and were clearly representative of the pleuropneumonia-like group of organisms.

Biological Characteristics of the Associated Pleuropneumonia-Like Organisms

The presence of pleuropneumonia-like organisms in washings and cultures from the eyes of white mice has been reported by several workers.

In 1937 a communicable disease of white mice, termed infectious catarrh, was described by the writer (2). Its etiology was attributed to minute, pleomorphic, Gram-negative cells which were descriptively referred to as "coccobacilliform bodies." The term "pleuropneumonia-like organisms," originally introduced by Klieneberger (3) in 1935, has now become adopted, at least tentatively, as the name for bacteria of this particular morphologic type. Although the coccobacilliform bodies which were originally isolated from chickens (4), then from mice (2), and later from rats (5), showed certain cultural differences from the bacteria whi·h are now recognized as pleuropneumonia-like organisms, the similarity between the two types of agents is so close that there is little reason for separating them.

Infectious catarrh was originally observed in 1935 in a group of Swiss mice which had been introduced from an outside source for temporary use (2). All experimental studies were made, however, in non-Swiss mice of the Princeton strain. Two forms of conjunctivitis were observed in the latter mice, the signs of the disease, as described, being identical with those in the present outbreak. One form was present in uninjected mice removed directly from the breeding colony. The second form, which differed from it only in the presence of polymorphonuclear leucocytes and coccobacilliform bodies in films from the eye, was observed in mice which had been injected intranasally with catarrhal exudates. The minute bodies were present in considerable numbers in the large epithelial cells and it was assumed that they were identical with those associated with infectious catarrh. A photomicrograph which was presented at that time is essentially duplicated by Fig. 1 of the present paper.

The coccobacilliform bodies described in 1937 from mice with infectious catarrh were cultivable only in the presence of substances extracted from 10-day chick embryo tissue. There was no evidence of growth in fluid, defibrinated horse blood at the base of slanted nutrient agar, the only medium containing blood which was then used. A second organism which was not pathogenic for mice was found to be irregularly present however in this medium, on inoculation with catarrhal exudates, and was readily maintained by transfer. This organism which resembled the coccobacilliform bodies in size and shape but formed large compact clumps on cultivation was referred to as the X bacillus (2).

Sabin (6), in 1939, noted that mice injected intraocularly with certain exudates (rheumatic

fever) and also with broth and normal synovial fluid yielded positive pleuropneumonia-like cultures from the eye with great regularity. Nutrient agar enriched with approximately 30 per cent horse serum was commonly used by this time for the cultivation of these bacteria. With the Rockefeller Institute (New York) Swiss stock at least 4 of each group of 6 mice (3 weeks old) gave positive cultures in ten different experiments. Further studies indicated that pleuropneumonia-like organisms are commonly found on the external surface of the eye under natural conditions. They are often present in large numbers and unassociated with other bacteria. Two other strains of mice, one Swiss and one non-Swiss, showed a similar condition but with a lower rate of carriage. Evidence was also obtained that the pleuropneumonia-like organisms were present on the mucosa of the nose and the accessory sinuses.

Additional observations of Sabin and Johnson (7) in 1940 indicated that the carriage of pleuropneumonia-like organisms by mice was probably not a transitory phenomenon, 6 strains being isolated from the conjunctiva and nasal mucosa of 4 out of 10 mature mice (6 months or older). It was believed probable that the carrier state was the result of contact infection acquired after birth, a positive culture being obtained from the nose of 1 out of 20 5 day old mice from infected mothers.

The morphologic and cultural characteristics of the pleuropneumonia-like organisms associated with the present outbreak of conjunctivitis and termed the "conjunctival strain" have been studied in some detail and compared with those of a strain associated with respiratory disease in mice and designated the "catarrhal strain." Comment will be made only on differences in behavior of the two strains. The methods used were a duplication of those reported by us (8) and will not be redescribed.

Serum-agar plates inoculated with eye exudates or with fluid cultures showed minute colonies which reached their maximum size on the 4th to the 5th day at 37°C. If the suspensions were undiluted most of the colonies were closely packed and ranged in size from 25 to 100μ, with only an occasional larger form. With dilute suspensions the colonies were well spaced and regularly larger, varying from 150 to 300μ in diameter. Examined by transmitted light at a magnification of × 100 the larger colonies were distinctive in appearance, showing a foamy internal structure composed of small vacuoles and granules. There was a tendency for these components to be arranged as irregular streamers which radiated outward from the center. A darker central area was sometimes observed but was not characteristic. The smaller colonies, under 100μ in diameter, commonly showed vacuoles and granules but not striations. In referring to these colonies, which were quite unlike the uniformly granular ones of the catarrhal strain, the term "radial type" will be used (Fig. 3).

Growth of the catarrhal strain in defibrinated horse blood at the base of slanted nutrient agar was followed by a slight degree of hemolysis. Films prepared from the fluid, 48 hours after inoculation, showed a sparse distribution of discrete bacteria. Growth of the conjunctival strain in this medium was not followed by hemolysis and in films the bacteria were arranged as tightly packed clumps of tiny spherical particles. These aggregates varied considerably in size, measuring up to 50μ in diameter, and were identical in appearance with those of the previously described X bacillus (2).

Microscopic preparations were made from 48 hour old horse serum–bouillon cultures of both strains. The bacteria were sedimented, washed once, and stained with Giemsa for 30 minutes. With pleuropneumonia-like organisms from cultures this method gave better results than did the Wayson stain. The conjunctival strain consistently showed a greater number of ring and small spherical forms with a corresponding decrease in the number of filaments and bacillary elements. In some preparations the latter forms were apparently absent.

Additional Observations on the Distribution of the Pleuropneumonia-Like Organisms in Mice

Cultural studies were made on several groups of mice of the Princeton strain to determine the relation of age and experimental handling to localization of the pleuropneumonia-like organisms and the relation of the latter to the periorbital nodules which occasionally accompanied the ocular reaction.

Examination of Adult Female Mice.—Pleuropneumonia-like organisms of the radial type were isolated from the eyes and the nasal passages of 18 (72 per cent) out of 25 females breeders, 8 to 10 months old. The amount of growth obtained from the nasal cultures was meager, the number of colonies being commonly less than 100. The lungs, middle ears, and nasal passages of these mice were normal. The eyes of 4 showed reduced signs of conjunctivitis.

Examination of Unweaned Mice.—No growth was obtained from the unopen eyes of 12 unweaned mice, 5 to 7 days old. Pleuropneumonia-like organisms were present in films from the eyes of the 5 breeders with which the young were in contact but signs of conjunctivitis were observed in only 1. Positive cultures were obtained from the open and inflamed eyes of 7 and from the nasal passages of 2 out of 14 unweaned mice, 15 to 18 days old. No growth was obtained from 6 with normal eyes and from 1 with conjunctivitis. Pleuropneumonia-like organisms were present in films from the eyes of the 5 breeders from the same cage. Two of these mice showed conjunctivitis.

Examination of Mice Injected Intranasally with Bacteria-Free Suspensions.—Radial type pleuropneumonia-like colonies were obtained from 31 (73 per cent) out of 42 nasal cultures and from 1 (2 per cent) out of 47 lung cultures from Princeton mice injected intranasally 4 to 5 weeks earlier with bacteria-free suspensions of rat lungs. The number of colonies varied from 10 or less (in 23) to 100 or less (in 9). Aside from conjunctivitis all these mice were normal at autopsy.

Examination of Mice Injected Intranasally with the Conjunctival Strain.—No growth was obtained from the lungs of 25 Princeton mice which had been injected intranasally 4 weeks earlier with eye exudate or cultures of the particular strain of pleuropneumonia-like organisms. None of these mice showed any involvement of the middle ears, lungs, or nasal passages. Fifteen (73 per cent) of the nasal cultures were positive but there was no apparent increase in the number of radial type colonies which varied from 10 to several hundred.

Examination of Mice Injected Intranasally with the Catarrhal Strain.—Mice injected intranasally with the catarrhal strain of pleuropneumonia-like organisms showed no apparent change in the behavior of these bacteria as a result of the superimposed eye infection. The nasal implantation was followed by a high rate of otitis media and lower rates of pneumonia and rhinitis. Fifty out of 54 middle ear cultures yielded a pure growth of the catarrhal strain, while 4 (7 per cent) showed a much scantier growth of the conjunctival strain.

Examination of Mice with Periorbital Nodules.—Twelve recently weaned mice in which the conjunctivitis was accompanied by unilateral nodule formation were removed from the breeding colony for examination. The position of the nodule in relation to the eye varied but more commonly was located along a line between the eye and the ear. Postmortem inspection indicated that these nodules, which ranged in size from 5 to 8 mm. with an elevation of 3 to 4 mm., were in effect subcutaneous abscesses containing a voluminous, thick, purulent material.

In the presence of penicillin a heavy pure growth of radial type pleuropneumonia-like colonies was obtained from the eyes of the 12 mice and a much scantier growth from 5 of the abscesses. In the absence of penicillin there was a dense growth of miscellaneous bacteria from both loci. Gram-negative bacilli, of which 3 cultural types were observed, predominated. Their distribution in each pair of cultures was usually the same but varied from mouse to mouse.

Transmission of the Pleuropneumonia-Like Organisms to Swiss Mice

The morbidity of conjunctivitis in mice of the Princeton strain has persisted at a high level over a period of 21 months. The manifestations of this condition were so commonly encountered in young mice and ocular carriage of the associated pleuropneumonia-like organisms was so high in older animals that it was impossible to use them in an experimental study of the disease. Attention was then focused on a colony of Swiss mice which has been maintained by this department for some years. It was found that these mice were neither subject to conjunctivitis, under the conditions which prevailed in the breeding colony, nor to carriage of the pleuropneumonia-like organisms.

The colony of normal Swiss mice is kept in a different building from that which houses the Princeton strain subject to conjunctivitis, but both are cared for by the same attendant. The size of the Swiss colony varies but it is generally operated with approximately 1000 breeders. The method of husbandry is the same with both colonies.

The Swiss mice were frequently inspected over a period of a year, particular attention being paid to the weaned young. Manifestations of the communicable conjunctivitis were never observed. By far the majority of the mice showed bright and wide-open eyes with no indication of local irritation. Occasionally a mouse with a distended and opaque eye, accompanied by increased lacrimation, was encountered but this condition was not transmissible and pleuropneumonia-like organisms were not associated with it.

Twenty-five Swiss mice, 4 to 5 weeks old, were removed at random from the cages of weaned young for cultural examination. In the presence of penicillin serum-agar plates inoculated with washings from the normal eyes of these mice were bacteriologically sterile.

Transmission by Direct Contact

The experimental transmission of conjunctivitis to Swiss mice was first attempted by direct exposure for varying periods to naturally infected Princeton mice.

In five experiments contact was established by placing 5 normal Swiss mice, 12 to 15 gm. in weight, in the same cage with an equal number of Princeton mice with acute conjunctivitis, and in one experiment with a single diseased animal. The originally infected mice were removed after an interval of 3 to 4 weeks and autopsied. Each group of the exposed Swiss mice was held under observation for an additional period of 1 to 24 days and then killed. At this time stained films or cultures were made from the eyes.

The results of the six experiments are summarized, in part, in Table I. Conjunctival films from all the 26 infected Princeton mice showed pleuropneumonia-like organisms together with numerous miscellaneous bacteria among which Gram-negative bacilli predominated. Marked signs of conjunctivitis were observed in 22. Pleuropneumonia-like organisms were also present in the eye washings from 29 of the 30 exposed Swiss mice (96 per cent). An ocular reaction, limited to increased lacrimation, was observed in only 4 (13 per cent). The eye films from these mice showed comparatively few secondary bacteria. During the intervening period between exposure and autopsy

a few additional Swiss mice showed a transient watering of the eye but at no time was there any evidence of an acute inflammatory reaction. At autopsy, however, 15 of these mice did show an increased volume of conjunctival fluid and this contained a few polymorphonuclear leucocytes. Seven of the 10 exposed mice of groups 4 and 5 yielded a scanty growth of pleuropneumonia-like colonies of the conjunctival type on culture from the nasal passages.

Stained films from the eyes of the 15 Swiss mice of groups 1, 2, and 3, made before contact was established, had shown no pleuropneumonia-like organisms. Films were again made on the 7th day of contact and at this time the organisms under consideration were present in 9. Two films from group 2 and 4 from

TABLE I

Results of the Exposure of Normal Swiss Mice to Princeton Mice with Conjunctivitis

Group No.*	Time between exposure and autopsy	No. of mice with		
		Conjunctivitis	Pleuropneumonia-like organisms	Leucocytes
	days			
1	36	1	5	3
2	22	0	5	2
3	31	0	5	2
4	57	1	5	4
5	58	1	4	2
6	28	1	5	2

* 5 mice were exposed in each group.

group 3 were negative. The presence of these bacteria was not accompanied by detectable signs of conjunctivitis.

A second transmission experiment involving contact between previously exposed and normal Swiss mice was begun with the 6th group of Table I and maintained for 10 passages.

Five normal Swiss mice were placed in the same cage with the 6th group of infected Swiss mice and left with them for 40 days. This procedure was repeated 10 times in all as indicated in Table II. At the end of each passage the previously exposed mice were autopsied and the the conjunctival washings examined microscopically or culturally. In some instances pleuro-pneumonia-like organisms were also looked for during the period of contact.

The results of this experiment are summarized in Table II. In all, 44 of the 50 Swiss mice (88 per cent) exposed to infection during the 10 passages showed pleuropneumonia-like organisms in the conjunctival washings. In most of these fluids they were numerous whereas miscellaneous bacteria were few in number and were generally limited to Gram-positive micrococci. The volume of the fluid was increased in 20 of the mice and polymorphonuclear leucocytes

were observed in 14. However, only 3 of the 50 mice (6 per cent) showed a definite conjunctivitis. A slight watering of the eye was sometimes noted during the period of exposure but was generally of transient duration. At autopsy the lungs, middle ears, and nasal passages of these mice were uniformly normal.

An additional contact experiment was carried out in Swiss mice to determine whether the disease and its associated bacteria were communicated by maternal transfer.

A normal, female, Swiss mouse with 6 nursing young, 7 days old, was placed in the same cage with 4 infected Princeton mice. Microscopic examination was made of conjunctival wash-

TABLE II

Results of the Exposure of Normal Swiss Mice to Swiss Mice Infected with Pleuropneumonia-Like Organisms and the Maintenance of Infection by Passage

Passage No.*	Time in contact	No. of mice with		
		Conjunctivitis	Pleuropneumonia-like organisms	Leucocytes
	days			
1	40	0	5	1
2	30	0	5	1
3	28	0	5	1
4	32	1	3	2
5	34	0	5	0
6	35	0	5	3
7	34	0	5	2
8	56	0	3	1
9	28	0	5	0
10	46	2	3	3

* 5 mice were exposed in each passage.

ings from both groups of mice and from 3 selected generations of Swiss mice which were subsequently reared from the young first exposed.

On the 15th day after contact was established pleuropneumonia-like organisms were demonstrable in eye films from the 4 Princeton mice, from the Swiss female, and from 5 of her 6 young. Pleuropneumonia-like organisms were also present in conjunctival washings from 2 of 5 mice in the first subsequent generation, from 3 of 6 in the second, and from 5 of 7 in the third. Approximately half of the eye films from the 25 exposed Swiss mice in this series showed a few polymorphonuclear leucocytes but only 3 of the mice (12 per cent) showed definite signs of conjunctivitis.

Transmission by Nasal and Conjunctival Instillation

The preceding experiments indicated that the exposure of normal Swiss mice to infected Princeton mice resulted in a high ocular carrier rate of pleuro-

pneumonia-like organisms but a low rate of conjunctivitis. Two series of experiments were conducted to determine the outcome of nasal and conjunctival instillation in relation to the pathogenesis of the disease and the establishment of the associated bacteria.

Three groups of 5 normal Swiss mice 12 to 15 gm. in weight were injected intranasally, using ether anesthesia, with approximately 0.05 cc. of a saline suspension of pooled conjunctival washings from 5 Princeton mice with conjunctivitis. Three additional groups were similarly injected with 48 hour old, 30 per cent horse serum–bouillon cultures of 3 radial type pleuropneumonia-like organisms recently isolated from the eyes of infected Princeton mice. The injected mice were inspected frequently for a period of 3 to 4 weeks. At autopsy Wayson-stained films of conjunctival washings from each mouse were examined microscopically. Thirty per cent horse serum-agar plates containing penicillin were inoculated with individual nasal and conjunctival washings and with a suspension of the pooled lungs from each of the 6 groups of 5 animals. The inoculated plates were incubated at 37°C. for 7 days and examined microscopically at a magnification of × 100.

The results of the 30 intranasal tests were completely negative. None of the injected mice showed any indication of conjunctivitis during the period of observation. At autopsy the eyes were uniformly normal and there was no involvement of the lungs, middle ears, or nasal passages. The conjunctival films showed no pleuropneumonia-like organisms and all cultures were sterile in respect to these bacteria.

Conjunctival instillations were made bilaterally in normal Swiss mice, using ocular washings from infected Princeton mice and recently isolated cultures of the pleuropneumonia-like organisms. Prior to injection the mice were anesthetized with ether and a small amount of the suspension was introduced under the eyelids with a fine capillary pipette. With each type of inoculum this was done only once in 10 mice but 5 times at intervals of 2 to 3 days in 6 mice. The injected mice were frequently inspected for a period of 2 to 4 weeks and then killed. At autopsy pleuropneumonia-like organisms were looked for microscopically in conjunctival films and culturally on serum-agar plates inoculated with individual ocular and nasal washings and with pooled lung suspensions from each group of mice.

Negative findings were again obtained from the Swiss mice which had received a single conjunctival instillation of ocular washings and a single and multiple instillation of pleuropneumonia-like organisms in culture. The 26 mice used in these 3 tests showed no signs of conjunctivitis during the period of observation or at autopsy. The lungs, middle ears, and nasal passages were normal. Pleuropneumonia-like organisms were not demonstrable in films or in cultures from the eyes, nasal passages, or lungs.

The 6 Swiss mice which had received multiple instillations of ocular washings showed no persistent signs of conjunctivitis either during the period of treatment or subsequent to it. A transient watering of the eyes was noted in 2 of the mice. Numerous pleuropneumonia-like organisms were observed, however, in stained conjunctival films made 7 to 14 days after the last instillation. Leucocytes were present in 4 of the films examined on the 7th day and in 3 on the 14th day. Cultures of ocular washings which were made from

each of the 6 mice on the 14th day yielded a heavy growth of pleuropneumonia-like organisms. Cultures from the nasal washings were likewise positive but the number of colonies was much reduced.

DISCUSSION

The biological characteristics of the pleuropneumonia-like organisms isolated from the eyes of mice with conjunctivitis were sufficiently uniform and distinctive to warrant classifying them as a special strain. The most significant difference between these organisms and the ones associated with infectious catarrh was that of their localization in the host. The conjunctival strain was essentially restricted to the appendages of the eye and the nasal passages, whereas the catarrhal strain tended to multiply throughout the respiratory tract and particularly in the middle ears. The sparse growth which was commonly obtained in cultures from the nasal passages of mice with conjunctivitis suggested the survival of organisms carried there from the eye rather than actual multiplication.

It is probable that the ocular inflammation which was observed in 1935 (2) in mice with infectious catarrh was identical with the presently described conjunctivitis and that the so called X bacillus which was encountered in horse blood agar cultures was actually a pleuropneumonia-like organism of the conjunctival strain. The relation of this strain to the pleuropneumonia-like organisms isolated by Sabin (6) from the external surface of the eyes of supposedly normal mice was less obvious. The morphologic and colonial characteristics of the respective organisms were somewhat different and the presence of those which he described was apparently not accompanied by conjunctivitis.

Observations on the mode of transmission indicate that conjunctivitis and the associated pleuropneumonia-like organisms are both acquired by direct ocular contact with fluids discharged from the eyes and nasal passages of infected mice. It is evident that conjunctival implantation of the organisms regularly precedes their nasal establishment. Ocular carriage of these bacteria occurs in a high percentage of adult animals though often in the absence of detectable signs of conjunctivitis. Unweaned mice are exposed to the disease by direct contact with adults and, as noted earlier by Sabin and Johnson (7), some of them become infected during the ensuing period of nursing. It is probable, however, that ocular development of the pleuropneumonia-like organisms does not occur until after the eyes are open. An additional opportunity for spread of the infection comes at the time the weaned mice are brought together from different breeding cages. The combined periods of exposure, before and after weaning, ultimately result in a morbidity rate which must be close to 100 per cent.

The outcome of the transmission experiments in Swiss mice brings further evidence that the pleuropneumonia-like organisms associated with conjunc-

tivitis have special affinity for the conjunctival mucosa. The low rate of ocular reaction detectable microscopically in mice infected with these organisms was unexpected and afforded only partial support for their conjectured etiological relation to the disease. It is of interest in this connection that the high recovery rate of pleuropneumonia-like organisms reported by Sabin (6) from the external surface of the eyes of Swiss mice was apparently not accompanied by manifestations of conjunctivitis.

In view of the ease with which pleuropneumonia-like organisms were recovered from the conjunctiva of naturally and experimentally exposed mice it is of interest that the organisms were so difficult to establish by artificial instillation. It is probable that many were rapidly eliminated from the conjunctiva by mechanical or biological means and that persistence may occur only after repeated implantation. The pleuropneumonia-like organisms which survived artificial introduction were implanted by the multiple conjunctival instillation of undiluted ocular washings. These results suggest that conjunctival establishment of the organisms, under natural conditions, is brought about by repeated contact aided by the presence of some protective substance, as mucin, in the conveying particles or droplets.

An attempt has been made to rear a group of infection-free mice of the Princeton strain. Several generations of specially selected mice have now been under observation for a period of 6 months and have shown no indication of ocular or nasal carriage of pleuropneumonia-like organisms. It is hoped that they can ultimately be used to study more conclusively the relation of these bacteria to conjunctivitis.

SUMMARY

An outbreak of conjunctivitis, unaccompanied by involvement of the respiratory tract, is reported in a colony of white mice.

A special strain of pleuropneumonia-like organisms was regularly isolated from the eyes and nasal passages of affected mice but not from the lungs or middle ears.

Ocular carriage of these organisms in the absence of an inflammatory reaction occurred in at least 50 per cent of the adult mice.

Transmission to the young was presumably initiated by parental contact, the organisms being recoverable after the eyes were open, and was continued after weaning by direct contact between cage mates.

These organisms were repeatedly established on the conjunctiva of normal Swiss mice by direct contact with infected animals and subsequently maintained there for ten successive passages.

Multiplication of the pleuropneumonia-like organisms, which was largely limited to the eye and its appendages, was accompanied by a low rate of conjunctivitis.

The multiple conjunctival instillation of ocular washings from infected mice was the only additional method of implantation of the organisms which was successful.

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EXPLANATION OF PLATE 15

Photographs by Mr. J. A. Carlile.

Fig. 1. Discretely arranged pleuropneumonia-like organisms in large epithelial cell from the conjunctiva of an infected mouse. Wayson stain. × 1560.

Fig. 2. Colony-like groupings of the organisms in a similar cell. Wayson stain. × 1560.

Fig. 3. Colonies of the conjunctival type of pleuropneumonia-like organisms. 4 day old serum-agar plate. × 100.
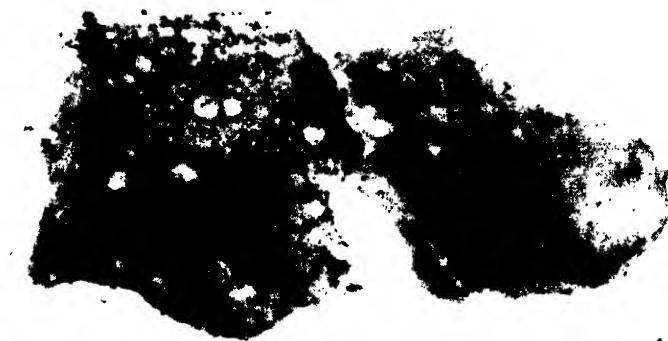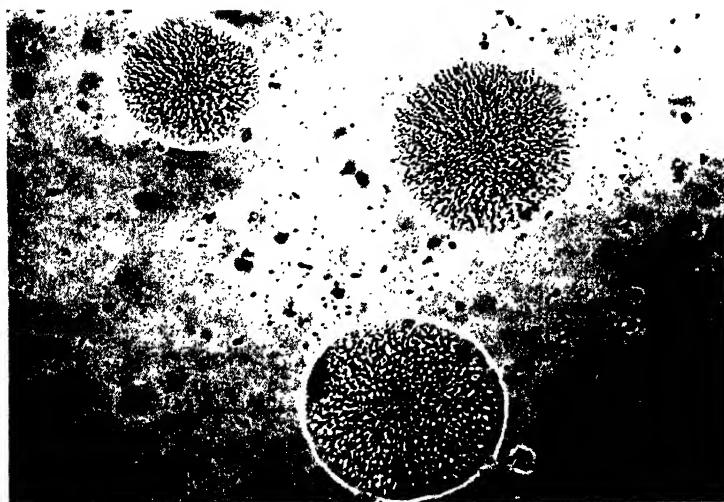
(Nelson: Pleuropneumonia-like organisms and conjunctivitis)

THE INTERACTION OF TOBACCO MOSAIC VIRUS AND OF ITS DEGRADATION PRODUCTS WITH DYES

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When tobacco mosaic virus is denatured by heating, it splits into two components, a nucleic acid and an insoluble protein. As will be shown, the intact virus exhibits entirely different staining properties from those of the insoluble protein and of the nucleic acid. The staining properties of the nucleic acid depend very much on its state of degradation. A comparison is also made with the staining properties of serum and of egg albumins.

Staining of the Intact Virus

At pH values above its isolectric point [pH 3.2–3.5 in buffers (1, 2) and pH 3.9 in water (3)], tobacco mosaic virus is negatively charged and, therefore, should combine with basic dyestuffs by virtue of Coulombic attraction. A study was made of the interaction of the basic dye acriflavine (trypaflavin) with the virus at a pH value above the isoelectric point of the virus.

The tobacco mosaic virus was purified by differential centrifugation by the method of Stanley (4), and the purified virus was then resuspended twice in water. The aqueous solution of the virus had a pH value of about 6.5 and an electrical conductivity corresponding to a NaCl solution of about 10^{-4} M. The binding of the dye to the virus was determined by sedimenting the virus from a solution containing the dye. The amount of dye bound by the virus is taken as the difference between the amount of dye originally present and the amount in the supernatant fluid after centrifugation (free dye). Fourteen Lusteroid centrifuge tubes containing 5 ml. of virus solution (2.55 mg. protein/ml.) and 5 ml. of dye solution were centrifuged for 1 hr. at 30,000 r.p.m. in a Bauer-Pickels air-driven centrifuge. The virus-free supernatant fluid was removed and the dye concentration was determined by measuring the optical density at λ = 446 mμ, the wave length of maximum light absorption of the dye, in a Beckman spectrophotometer and compared with an optical density-concentration calibration curve for the dye. It was found that the centrifuge tubes were not stained by the dye nor did any dye sediment in a virus-free solution. The experiments of absorption of the dye by the virus were made in water, 0.01 M NaCl and 0.10 M NaCl. In Fig. 1 are illustrated the experimental results; the moles dye bound per unit weight of virus

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vs. concentration of free dye. In water, the dye bound follows a Langmuir isotherm (5). Such a curve is expected on purely statistical grounds if it is assumed that the molecules adsorbed do not interact with one another (6) and has been observed for the methyl orange-serum albumin system by Klotz and his coworkers (7). Klotz presents his data as the reciprocal of the quantities given in Fig. 1 [see also Klotz (8)].

· The amount of dye bound/g. of virus is equal to $\alpha c/(K + c)$, where c is the molar concentration of free dye, α is the limiting value of the binding for high dye concentrations, and K is the dissociation constant of the dye ion and the charged group on the protein with which it binds. From the curve for water of Fig. 1 it is seen that the limiting value of binding, α, is equal to 1.3×10^{-4} M of dye/g. of virus. This value is identical with that obtained for the number of carboxyl groups of the virus in water at pH 6.5 as obtained by acid-base
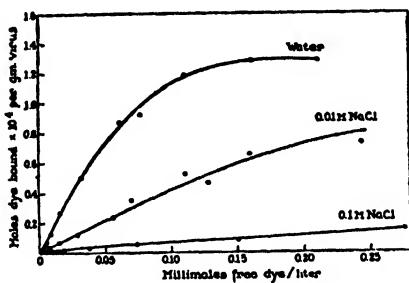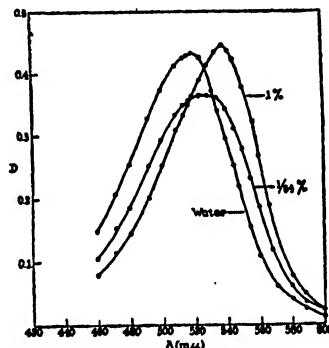
FIG. 1.   FIG. 2.

FIG. 1. The binding of acriflavine by tobacco mosaic virus.

FIG. 2. Absorption spectra of safranin (1.41×10^{-5} M) in the presence of varying concentrations of nucleic acid from tobacco mosaic virus.

titrations (3). Taking the molecular weight of the virus as 40,000,000, then the maximum number of dye molecules adsorbed on a single virus particle is 5.2 $\times$ 10^{3}. For low dye concentrations the amount of dye bound/g. of virus in water is given by $\alpha c/K$ and from Fig. 1, K is calculated to be equal to $10^{-4.1}$. The dissociation constant K is considerably increased in the presence of salts.

Methyl orange and rose bengal, both negatively charged ions, are not bound to the virus under the same experimental conditions as those performed with acriflavine.

Color Shift of Dyes With Virus Nucleic Acid

When a solution of the purified tobacco mosaic virus containing a small amount of salt is heated above 90°C., the virus breaks down into essentially two components—nucleic acid and an insoluble protein containing no phos-

phorus or carbohydrate (9, 10). The nucleic acid has been shown by Loring (11), by a chemical analysis of the carbohydrates and the purine and pyrimidine bases, to be of the ribose type.

In the present work the virus nucleic acid was obtained and purified by the method of Cohen and Stanley (10), and the insoluble protein was washed several times with 0.1 M NaCl, and then with water, and was saved for experiments to be described in the next section of this paper. These nucleic acid preparations had an intrinsic viscosity of 13.

In the presence of the virus nucleic acid, at pH 7.0, several dyes, including toluidine blue, methylene blue, and safranin, showed an alteration in the color of the dye from that which it possesses in water alone. In Fig. 2 is illustrated the absorption spectra (absorption given in terms of the optical density, D) of safranin in the presence of varying amounts of virus nucleic acid. The color shift, which can easily be observed by eye, varies from a maximum of 520 mμ (orange colored) for no nucleic acid present to a maximum of 540 mμ (red colored) for large amounts of nucleic acid present. For low nucleic acid concentrations, the spectrum is a composite of the altered spectrum of the dye bound plus the spectrum of the free dye in solution. The results of Fig. 2 are identical with those found by Michaelis (12) for phenosafranin in the presence of yeast nucleic acid.

The interaction of the dye with nucleic acid is influenced by high salt concentrations. Thus, if NaCl is added to the safranin-nucleic acid mixture in excess of 0.4 M, the color shift due to the nucleic acid is somewhat depressed. This may be due to a competition between sodium ions, which are present in large excess, and the basic dye cation for binding with the phosphate groups of the nucleic acid, or it may be due to incipient salting-out of the dye (12). However, since the salt concentration dealt with in this paper is equal to 0.1 M, where this salt effect is not detectable, we shall not consider this factor further.

By far the most important factor influencing the dye-nucleic acid interaction is the state of degradation of the nucleic acid. Thus, if the solution of safranin and the virus nucleic acid is kept at pH 11.9, where the nucleic acid is degraded, the solution exhibits the color which the dye has at this pH when no nucleic acid is present (Fig. 3). The virus nucleic acid is apparently also degraded by ribonuclease, since this nucleic acid at pH 7 to which crystalline ribonuclease[1] was added, failed to shift the color of safranin in water (Fig. 3). Yeast nucleic acid showed a similar behavior in the presence of ribonuclease. Kunitz (13) has shown that yeast nucleic acid is broken down by the action of ribonuclease into fragments one-eighth the size of the molecule and a similar result might be expected for the virus nucleic acid.

[1] We are indebted to Dr. M. Kunitz for providing us with this enzyme and with the desoxyribonuclease described below.

The color shift of safranin is also suppressed when thymus nucleic acid is subjected to analogous treatment. Thus, thymus nucleic acid causes the spectra of safranin to shift from 520 mμ to 540 mμ, but, if the solution is brought to pH 11.9, the color returns to that for the dye in water alone. A similar suppression of the shift is obtained by adding desoxyribonuclease to the thymus nucleic acid. A 0.02% solution of thymus nucleic acid containing 0.0025 M MgCl$_2$ and a small amount of gelatin was made. As McCarty (14) has shown, MgCl$_2$ is necessary for desoxyribonuclease activity and gelatin aids in stabilization of the protein. The solution shows with 1.69 $\times$ 10^{-5} M safranin a maximum
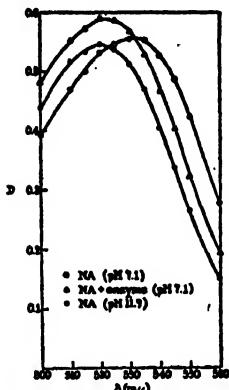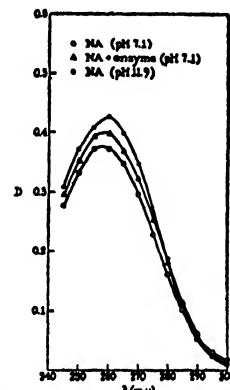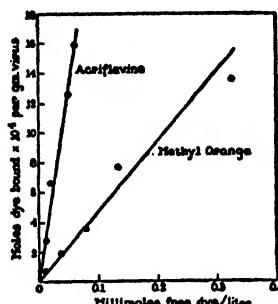
FIG. 3.   FIG. 4.   FIG. 5.

FIG. 3. Absorption spectra of safranin (1.69 $\times$ 10^{-5} M) in the presence of nucleic acid (0.035%) from tobacco mosaic virus with and without crystalline ribonuclease (2.1 γ/ml.) at pH 7.0 and the nucleic acid at pH 11.9.

FIG. 4. Ultraviolet absorption spectra of nucleic acid (0.0022%) from tobacco mosaic virus with and without crystalline ribonuclease (2.1 γ/ml.) at pH 7.0 and the nucleic acid at pH 11.9.

FIG. 5. The binding of methyl orange and acriflavine to the insoluble protein obtained from tobacco mosaic virus by heat denaturation.

at 540 mμ typical of nucleic acid (regardless of source). To this solution was added 50 γ of crystalline dexosyribonuclease, which has recently been crystallized by Kunitz (15), and the dye was added after 15 min. digestion. The maximum in the spectra of the dye moved down to 524 mμ, $i.e.$, toward its value in water. Similar experiments were performed with yeast nucleic acid and the virus nucleic acid, and also using ribonuclease under the same conditions given above. It was found that ribonuclease had no effect on thymus nucleic acid but does act on yeast nucleic acid and on the virus nucleic acid. It was also found that desoxyribonuclease has no effect on the yeast nucleic acid or on the virus nucleic acid.

Kunitz (16) has shown that the ultraviolet absorption at 260 mμ of yeast nucleic acid is increased when the nucleic acid is digested with ribonuclease. We have also found this result with virus nucleic acid (Fig. 4). A similar result is obtained by raising the pH of the virus nucleic acid solution to 11.9 (Fig. 4) and, therefore, it may be reasoned that the effect of the latter treatment involves a similar depolymerization process as that found by enzyme digestion (13).[3]

Since all the treatments of nucleic acid described above involve a breakdown into smaller molecules, it appears, as Michaelis (12) has suggested [see also Michaelis and Granick (17)], that the color shift of the dye in the presence of nucleic acid takes place when the dye cation combines with the highly charged phosphate groups which are arranged along the nucleic acid molecule. When the nucleic acid molecule is degraded, however, the dye behaves as though no nucleic acid is present.

Experiments similar to those described earlier for acriflavine were also carried out with safranin and the intact virus. As with acriflavine, safranin is bound to the virus and the amount of binding decreases with the addition of NaCl. In the presence of freshly purified intact virus, no shift in color is observed for safranin although the dye is bound to the virus particles. Thus, a 2% solution of the virus with $1.69 \times 10^{-5} M$ safranin showed, when the optical density was corrected for the light scattered by the virus, the same absorption spectra as that of safranin in water alone. When the virus solution is heated and safranin added, however, a color shift becomes immediately evident. No color shift is observed when the virus is allowed to stand at room temperature for 3 days at pH 11.9, although nucleic acid is released by this treatment, but it is in the depolymerized state as discussed above.

The failure of the intact virus to show the typical nucleic acid staining reaction indicates that the phosphate groups of the nucleic acid in the intact virus are not available for chemical reactions. This findings is in agreement with Loring (18) that ribonuclease has no enzymatic activity for the intact virus.

Staining of the Insoluble Heat-Denatured Product

The insoluble protein residue obtained by the method described in the previous section was shaken up in 0.1 M sodium phosphate buffer at pH 7.0 and used in this form. Since the insoluble protein is easily sedimented by low speed centrifugation, the samples shaken up with the dye under investigation were sedimented in a clinical centrifuge at 1500 r.p.m. for 15 min. The concentration of the dye in the clear supernatant was determined colorimetrically in the Beckman spectrophotometer at a wave length corresponding to the maximum

[3] Recently one of us (G.O.) has found that there is a marked decrease in the infrared absorption peak at 1230 cm.$^{-1}$ on depolymerization of nucleic acid. This appears to be due to loss of the phosphoric ester linkage. The details will be published elsewhere.

in the absorption spectra of the dye. The concentration of the protein was determined by measuring the nitrogen content of sediments of the protein suspensions containing no dye.

It was found that all the dyes investigated are bound in great quantities to the insoluble protein. In Fig. 5 is shown the amount of binding as a function of free dye concentration. As can be seen, the amount of acriflavine bound to the insoluble protein greatly exceeds that bound to the intact virus under the same conditions. Furthermore, the acid dyes, methyl orange and rose bengal, are also strongly bound to the insoluble proteins but are not bound to the intact virus. Rose bengal is so completely bound that the data cannot be represented on the same scale as that of Fig. 5. For example, it was found that a suspension of 0.02 g. of protein/ml. with $7 \times 10^{-4} M$ rose bengal, when centrifuged, showed no dye in the clear supernatent. Dialysis experiments using sausage casings under the same conditions of salt, protein and dye gave the same results. Unlike the results of binding of acriflavine in water to intact virus, the curves of Fig. 5 do not show any saturation value of binding. This suggests that the binding of the dye to the insoluble protein does not take place on specific amino acid residues, since there is not enough of one kind of amino acid residue in tobacco mosaic virus [see the amino acid analysis of Knight (19)] to account for such great binding. Probably no multilayer absorption of the dye or sticking of dye in the form of micelles is taking place since then theory shows (see, for example, Brunauer (20), Chapter 6) that the curve of the amount of dye bound vs. free dye would curve upwards. It is interesting to note that methyl orange and rose bengal are negatively charged, yet bind strongly to the insoluble protein which carries a net negative charge. The electrophoretic mobility of the denatured protein in 0.1 M phosphate buffer at pH 7.0 as measured in a Kunitz-Northrop microelectrophoresis apparatus was found to be the same as that for collodion particles coated with the intact virus. It was further found that the isoelectric point of the insoluble protein in 0.02 Sorenson's citrate-HCl buffers is pH 3.45 ± 0.05, a value which is identical with that measured for the intact virus in these buffers (3).

The fact that the denatured protein is insoluble but possesses the same net charge as that of the intact virus suggests that the denaturation consists of a presentation, probably from the interior of the molecule, of hydrophobic side chain groups, which can bind with dyes by van der Waals forces. Our picture of the denaturation of the protein is contrary to that suggested by Mirsky and Pauling (21) and is closer to that suggested by Abramson, Moyer and Gorin [(22), p. 90]. Rose bengal, which contains much more highly polarizable groups, such as iodine and chlorine-substituted benzene rings, than do the other dyes studied, and which binds more completely than do the other dyes examined, indicates that van der Waals force is the predominating type of force responsible for this binding.

Egg albumin shows a similar reaction with rose bengal to that shown by tobacco mosaic virus. Thus, it was found from dialysis experiments that rose bengal is only slightly bound to native egg albumin in 0.1 M phosphate buffer at pH 7.0. If, however, egg albumin is denatured (becomes a turbid suspension at pH 7.0) by heating to 100°C., the dye is bound in large quantities. Incidentally, the isoelectric point of the denatured egg albumin as determined by microelectrophoresis is the same as that for the native protein.

Bovine serum albumin, on the other hand, exhibits an entirely different behavior. We found by dialysis experiments that rose bengal is adsorbed in large quantities to *native* serum albumin. This is consistent with the results of Klotz and his coworkers (7,8,23,24) who found that the acid dye, methyl orange, is bound to native serum albumin at pH 6.8. He and Walker (24) suggest that a necessary condition for the binding of methyl orange to serum albumin is the presence of the positively charged ϵ amino group of lysine, since binding is decreased in the region of pH 10 where the ϵ amino acid residue of lysine becomes electrically neutral. It is not clear, however, why other proteins, such as tobacco mosaic virus and egg albumin, which contain lysine, do not adsorb the dye in the native state. The unusual behavior of serum albumin is also manifested by its ability to combine, apparently by van der Waals forces, with fatty acids (25,26,27) and other substances (28,29) and by its unique physiological role in the blood stream (30).

SUMMARY

Acriflavine, a basic dye, binds to purified tobacco mosaic virus according to a Langmuir isotherm. The dissociation constant for the dye-virus complex is equal to $10^{-4.1}$ and a maximum of 1.3×10^{-4} moles of dye are bound/g. of virus.

The intact virus does not show the typical nucleic acid staining reaction (color shift with safranin and other metachromatic dyes). On heating the virus, however, the solution shows the nucleic acid staining reaction. The color shift does not take place when the nucleic acid is degraded by ribonuclease or by alkali. Changes in the ultraviolet spectrum of the nucleic acid are also observed on degradation.

The insoluble protein obtained by heating the virus binds strongly with several dyes. Egg albumin has some staining properties similar to those of the virus but serum albumin exhibits a different behavior.

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THE ULTRAVIOLET LIGHT AND PHOTOSENSITIZED INACTIVATION OF TOBACCO MOSAIC VIRUS

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INTRODUCTION

It has been known for some time that viruses are inactivated by ultraviolet radiation. The studies of Hollaender and Duggar (1) and of Price and Gowen (2) have shown that tobacco mosaic virus is inactivated according to the simple exponential law suggestive of a "one-hit" process. Rough calculations made by Uber (3) using the data of Price and Gowen indicate that the quantum yield for the inactivation process is much smaller than that observed for other proteins (4). Since tobacco mosaic virus is obtainable in pure form (Stanley (5)) and since considerable chemical and physical information is available concerning this nucleoprotein (for review, see references 6 and 7), it was felt that a quantitative study of the ultraviolet light inactivation of tobacco mosaic virus might be useful in an understanding of the general problem of the effects of ultraviolet radiation on living systems and on proteins.

Although tobacco mosaic virus is not affected by visible light, it can be rendered inactive if irradiated with visible light in the presence of a dye which is adsorbed on the virus. This photosensitization of the virus by the dye may serve as a model for elucidation of the photosensitized lethal action of visible light on simple biological systems.

In the present work, the quantum yield of ultraviolet light inactivation was determined. Possible physicochemical changes accompanying the inactivation were studied. The ultraviolet destruction of ribosenucleic acid obtained from the virus was also examined. The virus is inactivated by visible light when the dye acriflavine is adsorbed on the virus. The quantum yield of this process was determined and its effectiveness with and without oxygen was studied.

Experimental Methods and Results

Ultraviolet Light Inactivation of the Virus.—Solutions of tobacco mosaic virus purified by differential centrifugation by the method of Stanley (8) were treated with ultraviolet radiation of wave length of 253.7 mμ. The virus samples, between 4 and 5 ml.

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$(6.4 \times 10^{-4}$ gm. virus per ml. in 0.1 molar phosphate buffer at pH 7.0), were placed in quartz vessels and the samples were stirred during the period of irradiation (Fig. 1). An Hanovia mercury Sc-2537 lamp was used with an acetic acid (43 per cent solution) filter which cuts off radiation shorter than 253.7 mμ. Details of this filter system are given elsewhere (9). The samples received no heat from the lamp and remained at room temperature.
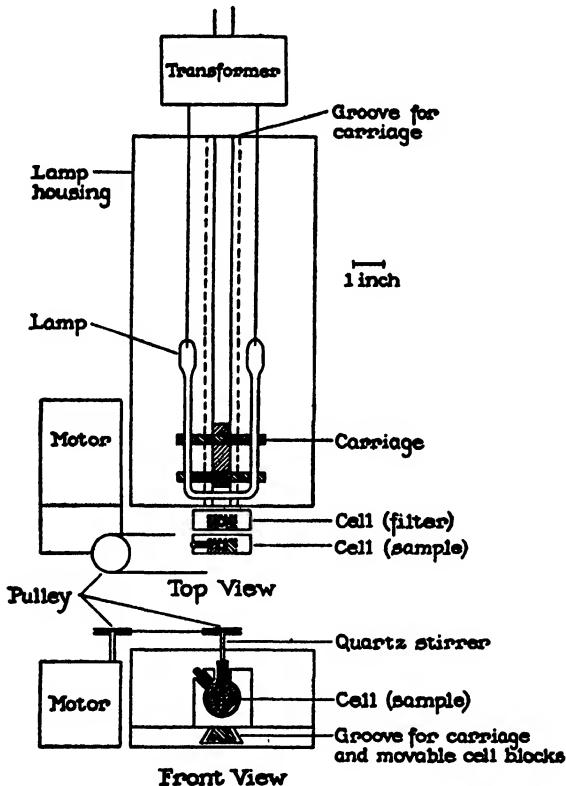
FIG. 1. Irradiation apparatus (see text for explanation).

At a distance of 3.5 cm. from the lamp, a fused quartz cell of 1.0 cm. depth and 2.6 cm. inside diameter was mounted in a 4.2 cm. square block drilled to fit and also mounted on a sliding rail. The quartz cell was equipped with two arms, each 0.6 cm. inside diameter, at an angle of 45° with each other. A fused quartz stirrer driven at 150 R.P.M. was employed. Between the lamp and the two-armed cell was placed, in a similar block, another quartz cell which contained the acetic acid.

The virus solution was irradiated for a certain length of time, the contents of the cell emptied, the next sample was irradiated for a shorter time, the cell emptied, and so forth. The intensity of light striking the contents of the cell was determined by a uranyl oxalate actinometer (10). With this actinometer the error in intensity at 253.7
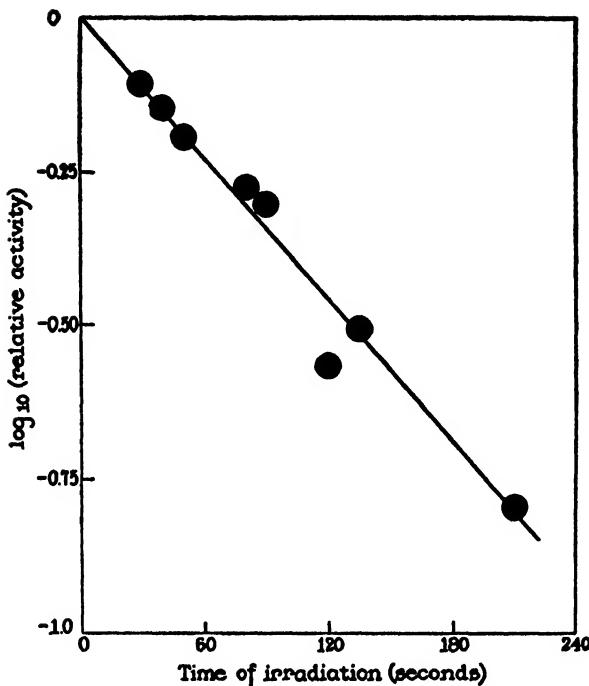
Fig. 2. Biological activity of tobacco mosaic virus as a function of time of irradia-
tion (253.7 mμ).

TABLE I

Biological Activity of Ultraviolet Light–Treated Tobacco Mosaic Virus

Time of treatment	Relative activity
sec.	*per cent*
0	(100)
30	78
40	71
50	64
81	53
90	50
120	27
135	31
210	16

mμ is not greater than 2 per cent of the absolute value (9). The virus concentration
employed corresponds, at 253.7 mμ, to a 96.8 per cent absorbing system. This wave
length is nearly that for the maximum in the ultraviolet absorption spectra for the
virus due primarily to the nucleic acid which it contains (6, 11).

The biological activity of the virus was determined on *Nicotiana glutinosa* by the

local lesion method of Holmes and others (12). The untreated virus was diluted to a virus concentration of 6×10^{-6} gm. per ml., and was applied on either half of the leaves and compared with the irradiated virus which had been diluted to give a comparable number of lesions on the other half. In Table I are given the mean values of three sets of determinations corresponding to nearly 20,000 lesions counted. Fig. 2 shows the logarithm (base ten) of the relative activity as a function of the time of treatment. The results follow the simple exponential decay curve.

The distance between the mercury lamp and the cell containing the virus was increased so that the intensity of the light on the cell was halved. Then the inactivation of the virus proceeded in twice the times given in Table I; *i.e.*, the inactivation depended on the dosage but not the intensity.

The quantum yield Φ for this process, that is the number of virus particles which are inactivated for each quantum of light absorbed, is defined as:

$$\Phi = \frac{\text{No. of moles virus particles inactivated per ml.}}{\text{No. of einsteins absorbed per ml.}}$$

$$= \frac{cf}{MEF}$$

(1)

c = concentration in gm. virus per ml.
f = fraction of molecules inactivated during time of exposure.
M = molecular weight of the virus.
E = einsteins falling on 1 ml. of the solution during exposure.
F = fraction of light absorbed by the virus at the given wave length, corrected for the average amount of light absorbed by the active virus.

In this study $c = 6.4 \times 10^{-4}$ gm. virus per ml. In 1 minute at a wave length of 253.7 mμ the fraction of virus inactivated (see Fig. 2) is equal to 0.409 (the remaining activity is 59.1 per cent). During 1 minute 2.37×10^{-7} einsteins fell on a milliliter of solution, as shown from actinometer measurements. The molecular weight of the active virus is now known to be 41 millions (7, 13) (Uber (3) took $M = 17$ millions, an earlier estimate now known to be incorrect.) At this wave length the optical density D is, for a solution of 6.4×10^{-4} gm. virus per ml., 1.50 corresponding to 3.2 per cent transmission or an opacity of 0.968.

Because of the large size of the virus particles, however, much of this opacity is due to light-scattering (14). Tobacco mosaic virus is known to contain about 6 per cent ribose nucleic acid (15). The nucleic acid accounts for nearly one-third of the optical density of the virus at 253.7 mμ. Other ultraviolet-absorbing constituents in the virus are, from the work of Knight (16), tryptophane (2.1 per cent), tyrosine (3.8 per cent), and phenylalanine (8.4 per cent). At 253.7 mμ these substances have molar coefficients (D/k where k is in moles per liter) of 3600 (in HCl), 400, and 200 respectively (data from Loofbourow (17)). Taking into account the concentrations of these ultraviolet-absorbing substances in the virus, we calculate that the optical density of the virus at

253.7 mμ should be about 50 per cent of that observed, indicating that the other 50 per cent of the optical density is due to light-scattering by the virus particles. A direct calculation of the light-scattering of the particles at this wave length is difficult because the particles are greater in length than the wave length of the light (18). However, with the use of light-scattering formulae (Equation 10 of reference 18) and using the index of refraction for visible light of the virus solution (14), nearly 80 per cent of the optical density of the scattered light may be accounted for (compare reference 19). It is difficult to correct for secondary scattering; i.e., light scattered by the particles which is then scattered and absorbed by the particles. We shall assume that the scattered light leaves the solution without being reabsorbed by the particles. Pertinent data for a solution of the virus of concentration 6.4 $\times$ 10^{-4} gm. per ml. are as follows:—

	Density	Transmitted	Opacity
		per cent	*per cent*
Observed.....................	1.50	3.2	96.8
Calculated from absorbing constituents....................	0.75	17.8	82.8

Since we are concerned with light absorbed by active virus, we must correct the absorption by the average value of the activity during the duration of the time considered. For a period of irradiation of 1 minute, this is seen graphically (Fig. 2) to be equal to 78 per cent. In summary, then, the fraction of light absorbed F equals opacity $\times$ fraction due to absorbing constituents $\times$ fraction absorbed by active virus (average value during 1 minute) = 0.968 $\times$ 0.85 $\times$ 0.78 = 0.64. Thus the quantum yield is

$$\Phi = \frac{cf}{MEF} = \frac{6.4 \times 10^{-4} \times 0.409}{4.1 \times 10^7 \times 2.37 \times 10^{-7} \times 0.64} = 4.3 \times 10^{-6}$$

Stanley (20) has performed qualitative experiments on the ultraviolet light inactivation of tobacco mosaic virus and found no measurable changes in certain chemical and serological properties of the virus. The increase in turbidity observed by Stanley (a full mercury arc was employed) was probably due to heat denaturation since in the present work it was found that irradiation of the solutions kept at room temperature produces inactivation without an increase in turbidity. Irradiation at 37°C. resulted in an increase in turbidity, however, although heating the normal virus at this temperature for the same length of time does not give an increase in turbidity.

In order to observe any possible chemical or physical modification of the virus, several tests were made on virus which had been subjected to ultraviolet light irradiation. It was found that a sample irradiated for as long as 30

minutes and having less than 2 per cent activity remaining showed no change in viscosity. Viscometric measurements give an indication of the shape of the virus (21), but here the intrinsic viscosity did not change under irradiation. This is in contrast to sonic irradiation which breaks down the rod-like virus particles (13). Other physical properties of the virus which remained unchanged were its moving boundary in the ultracentrifuge, its optical turbidity, its isoelectric point, its ultraviolet absorption spectra, and its appearance in the electron microscope. The irradiated sample was sedimented in the ultracentrifuge but no nucleic acid was found in the supernatant fluid. It was found, however, that the irradiated virus was more sensitive to heat denaturation than the untreated virus, but this change in property was not studied in any detail. This effect has also been found for proteins (22).

Inactivation of the virus by ultraviolet light irradiation was found to be independent of whether oxygen or oxygen-free nitrogen (prepared as described below) was bubbled through the system for 2 hours before and during the period of irradiation.

Degradation of Virus Nucleic Acid

Ribose nucleic acid is released from the virus on heating. Samples of nucleic acid made in this way and purified by the method of Cohen and Stanley (23) were found to have an intrinsic viscosity of 13, i.e. for a rod, an axial ratio of 10. This corresponds to "Nucleate C" of Cohen and Stanley and has, according to their measurements, a molecular weight of 15,000. This would correspond to about 12 tetranucleotides per nucleic acid molecule.

On irradiation of an 0.3 per cent solution of nucleic acid for 21 hours in a coil Sc-2537 resonance lamp with an acetic acid filter, the intrinsic viscosity decreased from the initial value to practically zero, suggesting that the molecules had broken down to much smaller molecules. This solution has no appreciable transmission and, therefore, only material in the outer surface of the vessel was irradiated; but due to the long exposure, convection currents and Brownian movement would be expected to allow all the molecules to be irradiated. It was necessary to use such a concentrated solution in order to make viscosity measurements.

It is known that degradation of ribose nucleic acid is accompanied by an increase in absorption at 260 mμ (24, 25). We found a 10 per cent increase in absorption (on suitable dilution) with the above solution of virus nucleic acid when it had been irradiated. A dilute solution of the virus nucleic acid (8 $\times$ 10^{-5} gm. per ml.; $F = 0.60$ at 253.7 mμ) was irradiated for 3 minutes at 253.7 mμ in the apparatus described in the previous section. The maximum in the ultraviolet spectra increased by 10 per cent in optical density. A further indication that the nucleic acid was degraded by the ultraviolet irradiation is that after irradiation, the nucleic acid failed to give the typical metachromatic staining with safranin (25).

Photosensitized Inactivation of the Virus

Tobacco mosaic virus particles are negatively charged at pH values above the isoelectric point of the virus and will, therefore, adsorb basic dyes. The basic dye chosen was acriflavine since its binding with the virus has been studied in detail (25). The spectra for this dye are given in Fig. 3 (filled circles). There are maxima at 450 mμ and 260 mμ; the former corresponding to that for the chromophoric group and the latter to that for the benzene rings. The dye
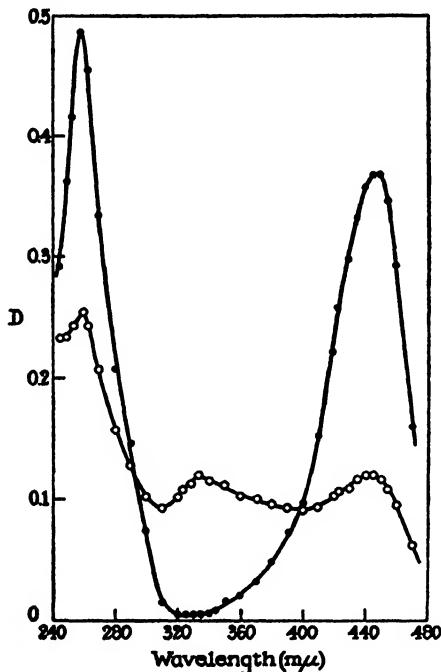
FIG. 3. Absorption spectra of acriflavine (8.3 $\times$ 10^{-6} molar) untreated (filled circles) and irradiated with blue light for 45 minutes (open circles).

and the dye-virus systems were irradiated with the 436 mμ line of mercury by placing in front of a mercury arc a blue glass filter transmitting this wave length. The lamp used was a water-cooled H-6 (General Electric) high pressure lamp. To further eliminate possible heating of the sample, a glass water bath was placed between the lamp and the filter. The sample was placed beyond the filter 8 inches from the lamp. The number of einsteins falling on a milliliter of solution per hour was 8.0 $\times$ 10^{-5} as determined by a uranyl oxalate actinometer.

The absorption spectra of the dye after 45 minutes' irradiation at 436 mμ are shown in Fig. 3 (open circles). Although the incident light corresponds to the region of absorption for the chromophoric group, the benzene rings are

also destroyed by the visible light and a new absorption peak is produced in the spectra at 335 mμ. The yellow fluorescence of the dye is also reduced on irradiation. In Fig. 4 (filled circles) is shown the decrease in absorption of the dye at 436 mμ as a function of time of irradiation. The optical density of the dye is proportional to its concentration and the decrease with time follows a first order reaction. In determining the quantum yield for the destruction of the dye, it is necessary to take into account the change in transmission of the solution, since as the color is lost there are less quanta absorbed by the solution. Then the quantum yield is given by the expression

$$\Phi = \frac{cf}{MEF'(1 - e^{-kt})}$$

where k is the rate of decrease of optical density. From Fig. 4 (filled circles) k equals 1 reciprocal hour (60 minutes to reduce the optical density to 1/eth of its value) so $1-e^{-kt} = 0.66$. For an optical density of 0.38 ($c/M = 8.3 \times 10^{-9}$) the fraction, F', absorbed in 1 cm. cell thickness is 0.24, so

$$\Phi = \frac{8.3 \times 10^{-9} \times 0.33}{8.0 \times 10^{-5} \times 0.24 \times 0.66} = 2.2 \times 10^{-4}$$

In the calculation of the quantum yield for the dye we did not take into account the fraction of the light which strikes the system and is transformed into innocuous yellow light by fluorescence. Taking a fluorescein solution of sufficient dilution (1.8 $\times$ 10^{-5} gm. per liter with a small amount of ammonium hydroxide) to give the same optical density as the acriflavine solution used, we find that at 436 mμ the acriflavine solution is one-half as fluorescent as is the fluorescein solution. Since at this dilution the quantum efficiency of fluorescence of fluorescein is nearly unity (28), then the efficiency of acriflavine is one-half; *i.e.*, half the blue light which strikes the solution is wasted as yellow fluorescent light which is not appreciably absorbed by the dye. The quantum yield calculation above must be corrected for this effect, that is, for the dye $\Phi = 4.4 \times 10^{-4}$.

In the presence of virus, the dye is not decomposed as rapidly as it is in the absence of the virus. In Fig. 4 (open circles) is shown the decrease in absorption at 436 mμ of the dye in the presence of the virus with the scattering due to the virus (6.4 $\times$ 10^{-4} gm. per ml.) subtracted. The destruction of the dye is approximately linear with time of irradiation.

The amount of dye adsorbed on the virus particles was determined by sedimenting the virus from the dye solution and measuring the loss of dye in the supernatant fluid (25). In the present case, a 10 ml. mixture of 8.30 $\times$ 10^{-6} molar dye and 6.4 $\times$ 10^{-4} gm. virus per ml. in distilled water gave in the supernatant fluid 5.80 $\times$ 10^{-6} molar dye; *i.e.*, 3.91 $\times$ 10^{-6} moles of dye were bound

to 1 gm. of virus. Taking a molecular weight of 41 millions for the virus particles, then there are 160 dye molecules adsorbed on each virus particle.

The inactivation of tobacco mosaic virus in the presence of the dye as a function of time of irradiation with blue light is shown in Fig. 5. The relative activities were determined by the half-leaf method described earlier. It will be seen that the inactivation is a first order reaction with a rate constant nearly that for the destruction of the dye in the absence of the virus (Fig. 4, filled
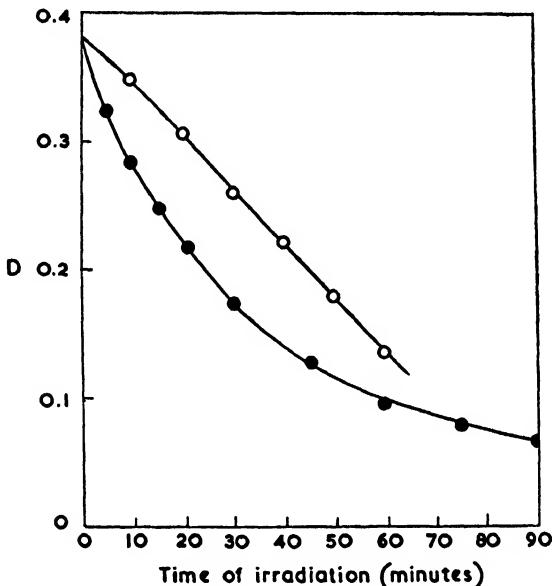
FIG. 4. Optical density at 436 mμ for acriflavine as a function of time of irradiation with blue light; dye alone (filled circles) and dye in the presence of the virus (open circles).

circles). No appreciable amount of inactivation of the virus takes place when a solution of virus in the absence of the dye is irradiated with blue light. The inactivation of the virus in the presence of the dye was considerably reduced when salt is added. Thus for the same dye and virus concentrations as above but in the presence of 0.1 molar NaCl, the relative activity after 1 hour irradiation was 88 per cent compared with 20 per cent when no salt was present. The intensity of fluorescence of the dye in the untreated sample was not influenced by the presence of the salt.

The destruction of the dye and the photosensitized inactivation of the virus are considerably influenced by the presence of oxygen, in complete contrast with the case of ultraviolet light inactivation. In Table II are shown the re-

sults on the photosensitized inactivation of the virus when bubbling air, oxygen, and nitrogen through the solutions. The gases were bubbled for 2 hours prior to and during the irradiation. The nitrogen was bubbled through Fieser's
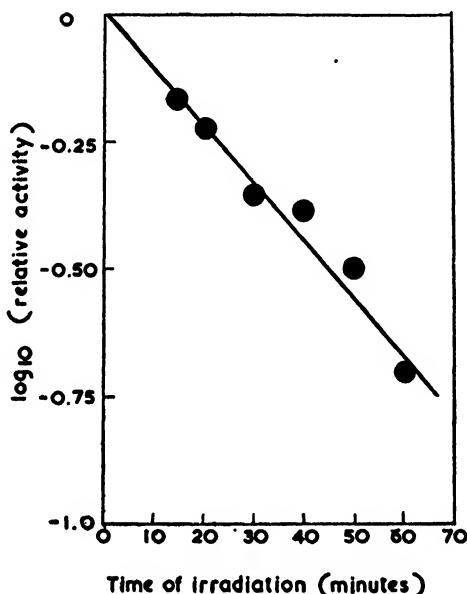
FIG. 5. Biological activity of tobacco mosaic virus in the presence of acriflavine as a function of time of irradiation with blue light.

TABLE II

Effect of Gases on Irradiation for 40 Minutes with Blue Light

	Control (unirradiated)	Nitrogen	Air	Oxygen
Density of dye at 436 mμ............	0.380	0.237	0.208	0.102
Relative activity of virus, *per cent*.....	(100)	50	42	Less than 1

solution and through lead acetate solution to remove traces of oxygen and hydrogen sulfide respectively (29).

DISCUSSION

Only light which is absorbed is effective in photochemical reactions; therefore, one must distinguish between decrease in transmission due to light-scattering and that due to consumptive absorption. For proteins which are small compared with the wave length of light, at 260 mμ the optical density due to scattering equals $3.3 \times 10^{-5} \, cM$ (Equation 10 of reference 18. Index of refrac-

tion increment for proteins about equal to 0.18). This is not an important factor for low molecular weight proteins (*e.g.*, a 1 per cent solution of molecules of molecular weight 35,000 gives an optical density due to scattering of 0.011), but for particles of virus dimensions the scattering constitutes an important part of the opacity of the system. For particles of sizes comparable with the wave length of light no simple formula for the scattering may be given, and it is simpler to subtract the density of the known absorbing constituents from the observed opacity of the system. With an exponential decline of active virus with time of irradiation at constant intensity, and with a first power dependence of rate constant on intensity, one may say that although every quantum absorbed does not cause inactivation, when inactivation does occur the primary process involves one quantum (22). In another form

$$-\frac{dV}{dt} = \Phi I_{abs.} \cdot \frac{V}{V_0} \tag{3}$$

where V/V_0 gives the fraction of the light absorbed at any time, assuming the molecular extinction coefficients of active and inactive virus to be identical (as observed for tobacco mosaic virus). $I_{abs.}$ is the intensity of light absorbed. Upon integration there results

$$\ln \frac{V_0}{V} = \frac{\Phi I_{abs.}}{V_0} t \tag{4}$$

For any given initial molar concentration V_0 we have

$$\frac{\Phi I_{abs.}}{V_0} = k_I \text{ (a constant)} \tag{5}$$

which shows that k_I is a pseudo first order constant which depends on V_0 (since $I_{abs.}$ is a function of V_0). For any initial virus concentration we have the well known formula

$$V = V_0 e^{-k_I t} \tag{6}$$

The quantum yield calculated by either Equation 1 or 4 is, of course, the same.

The question arises as to the meaning of the quantum yield 4.3×10^{-5} for the virus. Two mechanisms suggest themselves: (*a*) any highly, thermally excited portions of the molecule, subject to a Boltzmann distribution, may be sites of photochemical rupture; (*b*) localized sensitive groups are involved. The latter mechanism is more amenable to calculation. Considering this possibility we note that only one quantum out of 23,300 is effective. There are 13,000 aromatic residues per virus particle. Must some particular linkages be hit with a quantum yield around 0.5 or may any group be hit with a yield of 4.3×10^{-5}? It is pertinent to note that the quantum yields for rupture of the peptide

bonds (9), the disulfide bond (26), and a pyrimidine (27) are in the neighbor hood of 0.01 to 0.02. Since these values are much nearer 0.5 than 4.3×10^{-5}, it seems probable that 25 to 50 linkages are sensitive.

With regard to the degradation of the nucleic acid, we can make a rough estimation of the quantum yield in the following way. Assuming that a 10 per cent increase in light absorption by the dilute solution also means a breakdown to tetranucleotides, as apparently it does in the concentrated solution (see experimental), we calculate for a 3 minute exposure

$$\Phi = \frac{8 \times 10^{-6}}{\dfrac{15,000}{12} \times 3 \times 2.37 \times 10^{-7} \times 0.6} = 0.1$$

There are some reasons favoring a preponderance of depolymerization over oxidative degradation during irradiation of the free nucleic acid. (In the virus neither process takes place.) First, the viscosity, absorption, and staining properties of the irradiated nucleic acid are similar to those found after ribonuclease activity (25), a reaction typically considered as a depolymerization. Second, the above calculation assumes that the breakdown of purine and pyrimidine rings is slower than the depolymerization. This is probably correct, since the quantum yield for destruction of an ethoxy methyl amino pyrimidine was found to be only 0.018 (27). The approximate calculation above simply serves to show that the quantum efficiency for destruction of nucleic acid as well as for protein linkages is much higher than for the intact virus. The nucleic acid–protein framework of the virus apparently serves to hold together free radicals formed by quanta and thus allows for a high probability of recombination without net chemical change and with an over-all low quantum efficiency. The high stability of the virus may be contrasted with irradiation of enzymes which show relatively higher quantum yields accompanied by physical destruction of the molecules (22).

The mechanism of photosensitized inactivation is different from that of ultraviolet light inactivation. The former process requires the presence of a fluorescent molecule and is dependent on oxygen concentration (30), while the latter is a direct absorption mechanism proceeding at a rate independent of oxygen (22). Our data show that only adsorbed dye is effective, since, (a) the virus partially inhibits the destruction of the dye (Fig. 4, open circles) and (b) inactivation of the virus is slower if the dye is eluted by salt. In addition, if the fluorescent molecules were not attached to the virus particles they would lose their fluorescent energy to the solvent, since the lifetime of the dye is considerably less than the rate of diffusion of the dye to the virus.

The photosensitized inactivation of the virus proceeds under either oxygen or nitrogen; however, the effect is enhanced by oxygen. It is probably significant that the molar concentration of dissolved oxygen under an atmosphere of

pure oxygen or air is nearly that of the dye. This indicates that two mechanisms are operating, the one involving oxygen being the more efficient. The oxygen-dependent mechanism is perhaps related to similar effects observed with ionizing radiation (31).

SUMMARY

The quantum yield for the inactivation of tobacco mosaic virus has been determined at 253.7 mμ and found to be 4.3 $\times$ 10^{-5}. The possible significance of the observed one-hit process of inactivation has been discussed in terms of the kinetics and the rupture of model substances including nucleic acid. The ultraviolet light inactivation, which proceeds independent of oxygen, occurs without change in physicochemical properties, with the possible exception of an enhanced sensitivity to thermal denaturation.

The photosensitized inactivation of virus by acriflavine has been found to proceed parallel with the destruction of the dye. The action was found to be dependent upon adsorbed dye, and the inactivation is enhanced by the presence of oxygen.

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